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Growth Factor-Mediated Proliferation and Differentiation of Insulin-Producing INS-1 and RINm5F Cells: Identification of Betacellulin as a Novel β-Cell Mitogen*

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ABSTRACT

It is not clear which growth factors are crucial for the survival, proliferation, and differentiation of pancreatic β-cells. We used the relatively differentiated rat insulinoma cell line INS-1 to elucidate this issue. Responsiveness of the DNA synthesis of serum-starved cells was studied to a wide variety of growth factors. The most potent stimulators were PRL, GH, and betacellulin, a member of the epidermal growth factor (EGF) family that has not previously been shown to be mitogenic for β-cells. In addition to these, only vascular endothelial growth factor, insulin-like growth factor-1 and -2, had significant mitogenic activity, whereas hepatocyte growth factor, nerve growth factor-β, platelet-derived growth factors, basic fibroblast growth factor, EGF, transforming growth factor-α (TGF-α), neu differentiation factor, and TGF-β were inactive. None of these factors affected the insulin content of INS-1 cells. In contrast, certain differentiation factors, including nicotinamide, sodium butyrate, activin A, and 1,25-dihydroxyvitamin D_3 inhibited the DNA synthesis and increased the insulin content. Also all-*trans*-retinoic acid had an inhibitory effect on cell DNA synthesis but no effect on insulin content. From these findings betacellulin emerges as a novel growth factor for the β-cell. Half-maximal stimulation of INS-1 DNA synthesis was obtained with 25 pM betacellulin. Interestingly, betacellulin had no effect on RINm5F cells, whereas both EGF and TGF-α were slightly mitogenic. These effects may possibly be explained by differential expression of the *erb*B receptor tyrosine kinases. In RINm5F cells a spectrum of *erb*B gene expression was detected (EGF receptor/*erb*B-1, *erb*B-2/neu, and *erb*B-3), whereas INS-1 cells showed only expression of EGF receptor. Expression of the *erb*B-4 gene was undetectable in these cell lines. In summary, our results suggest that the INS-1 cell line is a suitable model for the study of β-cell growth and differentiation because the responses to previously identified β-cell mitogens were essentially similar to those reported in primary cells. In addition, we have identified betacellulin as a possible modulator of β-cell growth. (*Endocrinology* **139:** 1494–1499, 1998)

PANCREATIC islets are formed during fetal development from endodermal stem cells that lie in the ductal epithelium of the pancreas. This endocrine differentiation has classically been thought to be induced by interaction of mesenchymal cells with the adjacent ductal epithelium (1, 2). Recently, it has been shown that embryonic pancreatic epithelium can form islets even without contact with fetal mesenchyme. However, signals derived from the mesenchyme and extracellular matrix are important for the growth and development of the entire organ, including ducts and acinar components of the pancreas (3).

In the adult, the proliferative capacity of the islet cells is very limited, but islet mass can nevertheless increase through two possible mechanisms: replication of preexisting β-cells or differentiation from ductal precursor cells. β-cell replication can be readily demonstrated in fetal and neonatal islets both in rodents (4) and man (5). This activity decreases with aging, but a low degree of β-cell proliferation is present even in adult islets (6). It has been shown that islet-cell neogenesis can be reactivated in adult rodents, and that this is a major pathway responsible for the increased β-cell mass (7, 8).

The extracellular factors regulating islet differentiation and growth remain poorly understood. It is obvious that this requires the synergistic function of several genes and their products. Several growth factors have been associated with the regulation of β-cell differentiation, their replication and maintenance of β-cell mass (9). A recently identified exocrine pancreatic protein, INGAP (10), and other members of pancreas-associated lectin-type proteins, including reg (11), may be important for the initiation of ductal cell stimulation resulting in islet neogenesis. Some well-known factors including insulin-like growth factors (IGF-1 and -2), GH and related factors, particularly PRL (4, 12), are β-cell mitogens. Hepatocyte growth factor (HGF) is capable of increasing the proliferation of fetal human β-cells through its receptor, c-*met* (13). HGF also stimulates the growth of pancreatic duct cells, and may thus participate in the regenerative process at various levels (14). Vascular endothelial growth factor (VEGF) may have a role in β-cell maturation from ductal precursor cells because its receptor (Flk-1) is found in the ductal cells and exogenous VEGF stimulates their proliferation (15, 16).

Some members of the epidermal growth factor (EGF) family are proposed to have a role in the development of the

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endocrine pancreas. Transforming growth factor-α (TGF-α) is abundantly present in the developing pancreas (17), and its overexpression together with gastrin in transgenic mice increases the islet mass significantly (18). The EGF receptor (EGFR) is expressed throughout the human fetal pancreas, and mice lacking EGFR show disturbed formation of pancreatic islets (19). Betacellulin, a novel member of the EGF family, can together with activin A convert exocrine AR42J cells to insulin expressing cells (20). All growth factors of the EGF family are ligands for one or more of the four receptor tyrosine kinases encoded by the *erb*B gene family (EGFR/*erb*B-1, *erb*B-2/neu, *erb*B-3, and *erb*B-4) (21).

Rodent insulinoma cell lines are useful in studies of β-cell biology. Though these cells are often dedifferentiated, they share important characteristics with normal β-cells. The INS-1 cell line retains a relatively high level of insulin synthesis and release which is responsive to glucose (22). In the present study, we wanted to obtain a more complete picture of the growth factor mediated regulation of β-cells. For this purpose, we characterized the responses of INS-1 cells to several growth and differentiation factors.

Materials and Methods

Materials

Recombinant human IGF-1, recombinant human basic fibroblast growth factor (bFGF), recombinant human platelet-derived growth factors A/A and B/B (PDGF AA, PDGF BB), natural human transforming growth factor β (TGF-β), and recombinant human EGF were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human IGF-2, recombinant human betacellulin, recombinant human TGF-α and recombinant human VEGF were obtained from R & D Systems Europe, Ltd. (Oxford, UK). Recombinant human HGF was a gift from Dr. Jeffrey Rubin (Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD). Recombinant human nerve growth factor β (NGF-β), all-*trans*-retinoic acid, nicotinamide (NIC), and sodium butyrate (Sod.But.) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human neu differentiation factor (NDF) was a gift from Dr. Walter Birchmeier (MDC, Max Delbrück Centrum, Berlin, Germany). Recombinant human activin A (lot no. 15365–36) and recombinant porcine PRL were obtained through the National Hormone and Pituitary Program (NIDDK, NIH, Bethesda, MD). 1,25-dihydroxyvitamin D_3 (1, 25 -$(OH)_2D_3$) was a product of Leo Pharmaceutical Products (Ballerup, Denmark) and recombinant human GH (Genotropin) was from Pharmacia AB (Uppsala, Sweden).

Cell cultures

INS-1 cells (passages 70 to 95, kindly provided by Prof. Claes Wollheim, University of Geneva, Geneva, Switzlerland) were cultured in tissue culture flasks (Corning, NY) in complete medium composed of medium RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS (GIBCO, Life Technologies, Paisley, Scotland, UK) under a humified condition of 95% air and 5% CO_2 at 37 C (22). RINm5F cells (also from Prof. Wollheim) were cultured in medium RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS. For assays the cells were detached by a brief incubation with trypsin/EDTA (GIBCO).

DNA synthesis and cell proliferation

For proliferation studies, the cells (3×10^4) were plated in 96-well microtiter plates (Nunclon, Denmark) and allowed to attach in complete medium for 24 h. After that the cells were preincubated in medium supplemented with 0.5% FCS for another 24 h. The final 24 h incubation was carried out in medium supplemented with 0.5% FCS and the growth factor to be tested. For the measurement of DNA synthesis 1 μCi/ml of ^{3}H-thymidine (925GBq/mmol, 25Ci/mmol, Amersham) was added to each well for the last 4 h of culture. The cells were then harvested with trypsin/EDTA and transferred on glass fiber filters using a cell harvester (Skatron Combi, Skatron, Norway) and ^{3}H-radioactivity was measured in a liquid scintillation counter. The results are presented as percent of control ± SEM from three individual experiments done with six parallels.

To confirm the effect of mitogenic factors, the cells (1.5×10^5) were plated in 12-well plates (Falcon) in groups of three and incubated for either 4- or 7-day periods. After that the actual cell numbers were counted in a hemocytometer (Hawksley, UK). The results are expressed as cell numbers/well ± SEM from three experiments.

Measurement of insulin content

To determine the effect of the various factors on insulin content of INS-1 cells, the cells (1.5×10^5) were plated in 12-well plates and allowed to attach in complete medium for 24 h. After that the cells were incubated in complete medium with the factor to be tested for 7 days. The cells were then detached from the wells by cell dissociation solution (Sigma), washed with PBS (pH 7.4), resuspended in 300 μl of distilled water, and homogenized by sonication. Cellular insulin content was measured by RIA (Diagnostic Products Corp., Los Angeles, CA) in dilutions (1:20) of acid ethanol extracts. DNA was measured from the sonicates fluorometrically (23). The results are presented as percent of control ± SEM from triplicates of three individual experiments.

Analysis of gene expression

Total RNA from both INS-1 and RINm5F cells, as well as from rat pancreas, liver, and brain were isolated using guanidinium thiocyanate extraction followed by CsCl gradient centrifugation (24). Poly(A)RNA was purified from total RNA preparations with oligo(dT)-coated magnetic microbeads (Dynabeads, Dynal A.S., Oslo, Norway). Messenger RNA (mRNA) (5 μg/lane) was fractionated on a denaturating 1.2% agarose gel and then transferred to a nylon membrane (Hybond-N, Amersham) via capillary blotting. The complementary DNA (cDNA) probes were ^{32}P-labeled by a random hexamer priming method (Pime-A-Gene Labeling System, Promega). EGFR and *erb*B-2 (neu) cDNAs corresponded to the extracellular domains of the mouse and rat receptors, respectively. *erb*B-3 and *erb*B-4 cDNAs represented the whole coding areas of human mRNAs (all receptor cDNAs kindly provided by Dr. Päivi Miettinen and Prof. Kari Alitalo, University of Helsinki). Mouse β-actin cDNA used for loading control and mouse betacellulin cDNA (synthesized in our laboratory by RT-PCR) corresponded to the coding area of the mRNA. Hybridizations were carried out according to the Hybrid-Ease hybridization chamber instructions (Hoefer Scientific Instruments) at 65 C, buffer containing 1% SDS, 1 M NaCl, and 8% dextran sulfate. The blots were washed at 65 C in 1 × SSC and finally in 0.5 × SSC. Hybridization signals were visualized using a Bio-imaging analyzer (Fuji Photo Film Co., Ltd).

Statistical analysis

Significances of differences in DNA synthesis and insulin content were analyzed with one-way ANOVA and the Fisher's PLSD test using Statview 4.1 software (Abacus, Berkeley, CA) for the Macintosh.

Results

Cell replication

In the first set of experiments, we studied the effects of various factors on the DNA synthesis of serum-starved INS-1 cells. The concentrations used were expected to result in maximal receptor stimulation as based on previously published information on the bioactivity of the factors. Out of the several factors tested only GH, PRL, IGF-1, IGF-2, betacellulin, and VEGF increased the DNA synthesis (1.7- to 2.1-fold), whereas several others (*i.e.* HGF, NGF-β, PDGFs AA and BB, bFGF, TGF-β, EGF, TGF-α, and NDF) had no effect (Fig. 1).

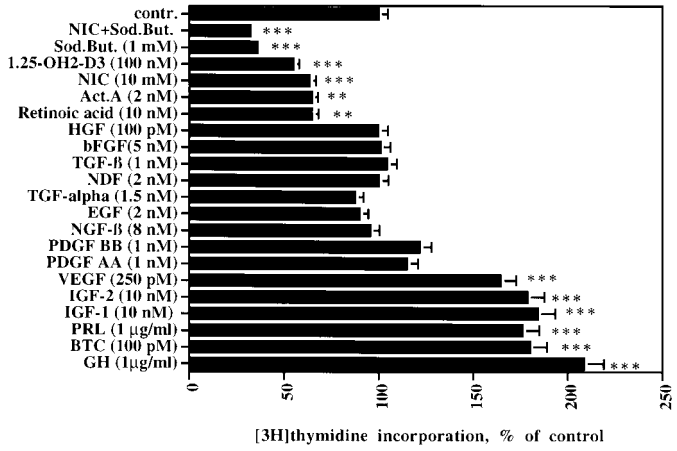

FIG. 1. Regulation of INS-1 cell DNA synthesis by growth and differentiation factors was determined by incorporation of ^{3}H-thymidine. Cells were stimulated with growth factors in medium containing 0.5% FCS for a 24-h period. ^{3}H-thymidine (1 μCi/ml) was added into the media for the last 4 h. Results are presented as percent of control ± SEM from three individual experiments done with six parallels (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

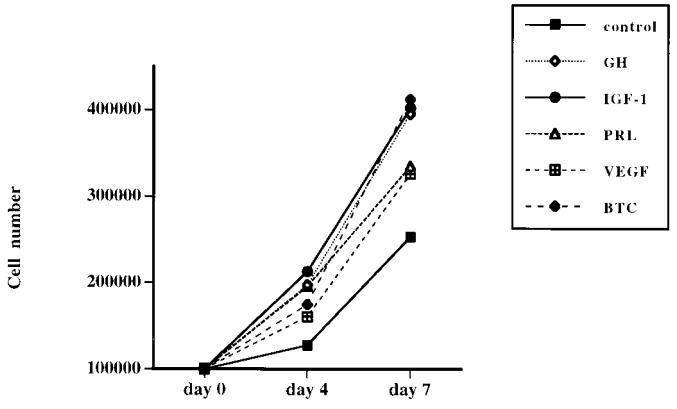

FIG. 2. Confirmation of mitogenic effects by cell counts. Results are expressed as means of the cell numbers from three experiments done as triplicates. At day 7, all of the growth factors had induced significant increases in cell number as compared with control ($P < 0.05$).

To confirm that increased thymidine incorporation actually represented mitogenic effects, cell counts were performed after 4 and 7 days of stimulation. All of the mitogenic factors, betacellulin, hGH, pPRL, VEGF, IGF-1, and IGF-2, significantly increased the cell number as compared with controls (Fig. 2). However, the magnitude of the increase in cell number was less than might be expected from the DNA synthesis results, suggesting that increased proliferation might also induce increased cell death.

We next compared the effects of EGF family members on INS-1 cells and on a less differentiated insulinoma cell line, RINm5F. The basal thymidine incorporation rate was over 1.5-fold greater in RINm5F cells than in INS-1 cells, indicating a more rapid growth rate of the RIN cells. In these studies, betacellulin was the only member of the EGF family that had a mitogenic effect on INS-1 cells. This effect was near-maximal at 200 pM and half-maximal at 25 pM concentration (Fig. 3A), whereas the other ligands (EGF, TGF-α, and NDF) had no response. Interestingly, the results were quite different in the RINm5F cells. In this cell line, betacellulin did not affect the DNA synthesis, whereas EGF and TGF-α had a slight but significant stimulatory effect (Fig. 3B).

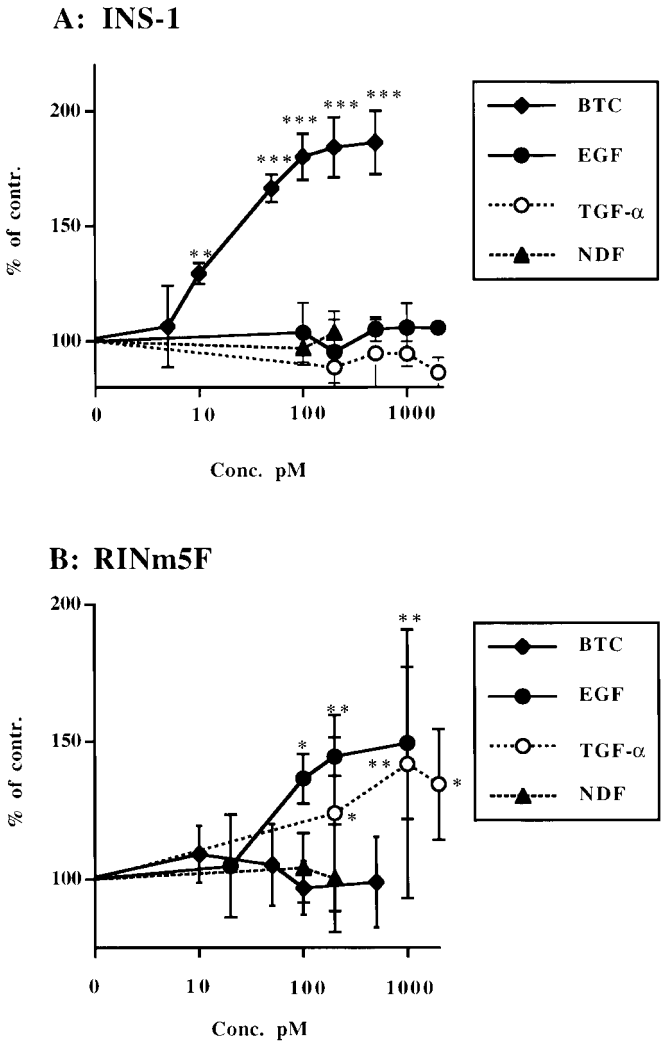

FIG. 3. Effect of the EGF family growth factors on the proliferation of RINm5F and INS-1 cells. DNA synthesis was determined as in Fig. 1. Results are presented as percent of control ± SEM from three individual experiments done with six parallels (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). The incorporation of ^{3}H-thymidine in controls was 1709 ± 79 cpm for RINm5F cells and 1130 ± 23 cpm for INS-1 cells.

Some growth inhibitory factors for INS-1 cell line were also detected. These included activin A, all-*trans*-retinoic acid, and 1,25-$(OH_2)D_3$. In addition, a pharmacological concentration (10 mM) of nicotinamide and a well-known differentiation factor, sodium butyrate (1 mM) also inhibited the DNA synthesis. The combination of nicotinamide and sodium butyrate was the most potent inhibitory condition for DNA synthesis (30% of control, Fig. 1). 1,25-$(OH_2)D_3$ also had a morphogenic effect on the INS-1 cells indicative of differentiation; it caused the cells to aggregate and detach from the culture dish into three-dimensional islet-like structures. This effect appeared within 48 h of stimulation and was maximal after 4 days.

Insulin content

The factors affecting proliferation were also tested for their effects on the insulin content of INS-1 cells (Fig. 4). After 7-day incubation with 10 mM nicotinamide, the cellular insulin content per DNA had increased 1.5-fold as compared with control. Incubation with 1 mM sodium butyrate had an almost 2-fold stimulatory effect. The combination of these two agents increased the amount of insulin per DNA 3.7-fold. Remarkably, 2 nM activin A was equally potent. 1,25-$(OH_2)D_3$ also increased the insulin content 1.8-fold. Retinoic acid did not affect the insulin content, neither did any of the mitogenic factors.

erbB expression

All known EGFR/*erb*B-1 splice variants (10 kb, 6 kb, 4.7 kb, and 2.7 kb) were expressed in both INS-1 and RINm5F cells. The level of expression was higher in RINm5F. Expression of *erb*B-2/neu (4.8 kb) was detected in RINm5F cells, and also strongly in rat pancreas, but there was no detectable expression of this gene in INS-1 cells. RINm5F cells also showed expression of *erb*B-3 (6.2 kb), which was not detected in INS-1 cells. No full-length transcripts of the *erb*B-4 gene were detected in either of the cell lines. A low level of betacellulin gene expression was detected in both cell lines (data not shown). As expected, insulin expression was clearly stronger in INS-1 cells (Fig. 5).

Discussion

We show that INS-1, a relatively differentiated rat insulinoma cell line, can be used as a model for studies of growth factor mediated regulation of β-cell growth and differentiation. We demonstrate that INS-1 cells respond to hGH, pPRL, and IGF-1 and -2, which have previously been identified as β-cell mitogens in primary cells, and also in INS-1 cells (4, 25–29). Moreover, we were able to identify interest-

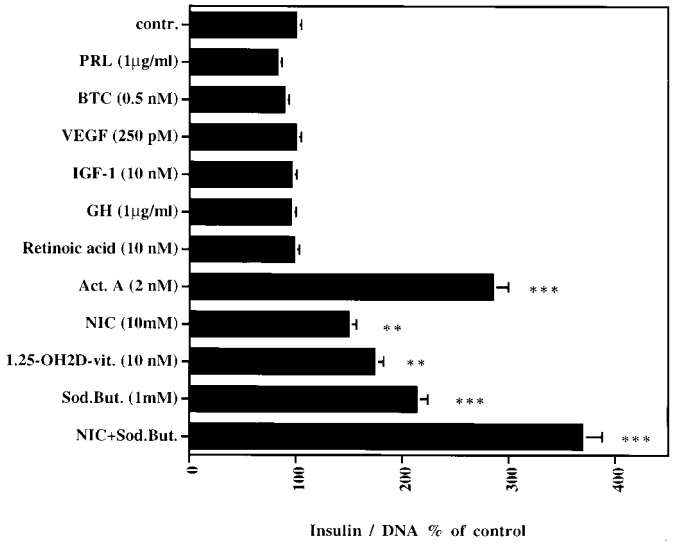

FIG. 4. Insulin content of INS-1 cells per DNA after 7 days of stimulation with growth and differentiation factors. Results are presented as percent of control ± SEM from three individual experiments done as triplicates (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

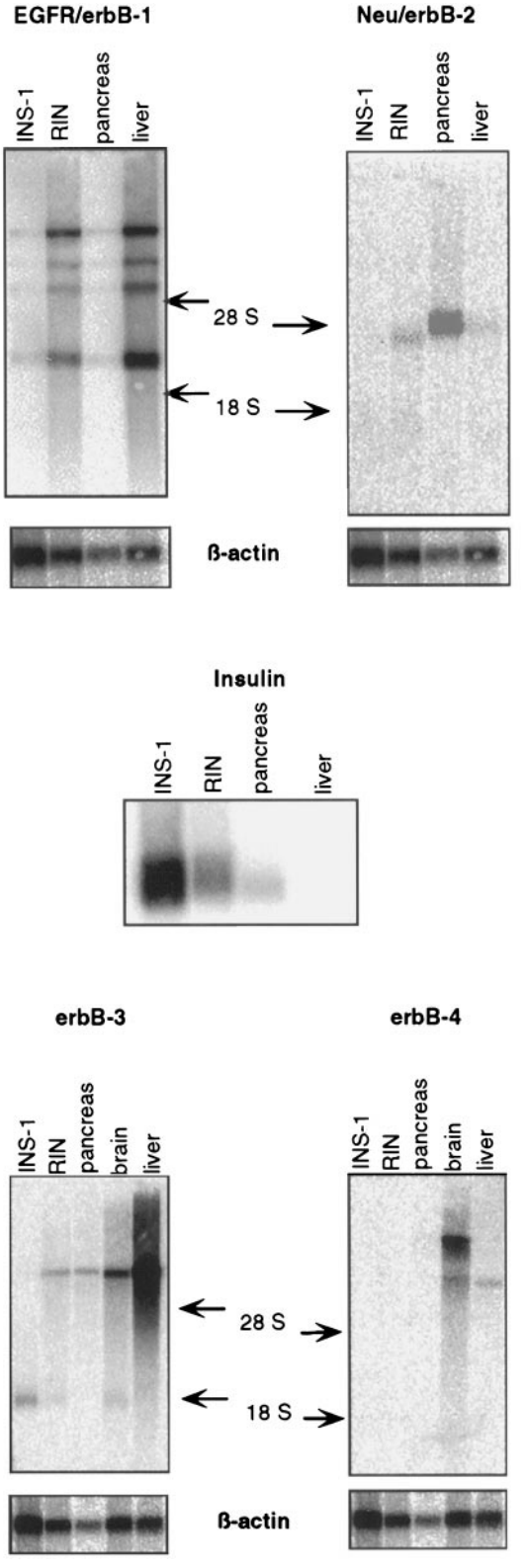

FIG. 5. *erb*B gene encoded receptor tyrosine kinase expression in INS-1 and RINm5F cell lines was detected by Northern blots using poly(A)RNA (5 μg/lane). Rat pancreas, liver, and brain were used as control tissues. *Arrows* indicate the 28S and 18S ribosomal RNA location. Actin expression was recorded as a loading control. The same filter used for *erb*B-1 and *erb*B-2 was hybridized with a rat insulin probe.

ing activities of other growth factors, possibly indicating physiological importance. We also identify a panel of growth-inhibitory substances, which are likely to play roles in β-cell differentiation.

Betacellulin, which was first discovered from mouse tumoral β-cells based on its mitogenic activities (30), stimulated INS-1 cell replication in picomolar concentrations. Betacellulin is a member of the EGF family, which consist of several polypeptide growth factors [*e.g.*, EGF, TGF-α, amphiregulin, heregulin (NDF) and heparin-binding EGF] (31). They are ligands for one or more of the *erb*B family receptor tyrosine kinases (EGFR/*erb*B-1, *erb*B-2/neu, *erb*B-3, *erb*B-4). It has been shown that betacellulin is a ligand for EGFR and *erb*B-4 (32). Signaling through the *erb*B-receptor tyrosine kinases is based on heterodimerization and cross-phosphorylation after binding of the various EGF ligands. Thus, although EGF has no affinity for *erb*B-2, EGF stimulates tyrosine phosphorylation of *erb*B-2/neu in cell lines bearing both EGFR/*erb*B-1 and *erb*B-2/neu. Likewise, betacellulin may cross-activate *erb*B-3 (21, 33, 34).

In our study, betacellulin was mitogenic only for the INS-1 cells but not for the more primitive RINm5F cells. On the other hand, EGF and TGF-α, which are well-known ligands for EGFR, did induce some proliferation in RINm5F cells but not in INS-1 cells. We found that these two cell lines express EGFR/*erb*B-1. However, only the more primitive RINm5F cell line expressed both *erb*B-2/neu and *erb*B-3 genes. *erb*B-4 expression was not detected in either of the cell lines. Low level of endogenous betacellulin expression was detected in both INS-1 and RINm5F cells. It is difficult to unambiguously explain the different response of the cell lines to the EGF family growth factors. One explanation might be that, though INS-1 cells show endogenous expression of betacellulin, the functional protein is not translated in these cells. The differential expression of *erb*B family receptors might also offer an explanation. *erb*B-2 has been shown to be the preferred heterodimerization partner of all other *erb*B proteins (35), and also *erb*B-3 tends to form heterodimers with EGFR/*erb*B-1. The absence of these two receptors in INS-1 cells would thus indicate that betacellulin activates only EGFR/*erb*B-1 in these cells, whereas in RINm5F cells a heterodimer of EGFR/*erb*B-1 with *erb*B-2 and/or -3 would be formed. Whether this is the explanation for the differential activity of betacellulin on these two cell lines, is not clear at this point. Alternatively, another unknown receptor for betacellulin could be involved in EGFR/*erb*B-1 heterodimerization and thus, in signal transmission (36).

The present observations on the specific mitogenic action of betacellulin on the INS-1 cells adds further proof for a specific role of this growth factor in islet development. This notion is based on the pattern of betacellulin expression, which is particularly high in the pancreas and small intestine (37). Additionally, betacellulin can specifically convert clonal exocrine pancreatic cells of the AR42J line into insulin-expressing cells (20). Betacellulin is also required for the induction of insulin gene expression in clonal α-cells transfected with the *pdx-1* gene (38).

Our results are in line with previous findings of GH and PRL being equally effective in increasing INS-1 DNA synthesis (28). Because the majority of hGH binding sites in both rat islets and in INS-1 cells are of lactogenic specificity (39), the effects of hGH may have been mainly mediated by PRL receptors.

VEGF was mitogenic for INS-1 cells in our experiments. Flk-1, a receptor for VEGF, has previously been identified in pancreatic ducts (15) and VEGF has been suggested to stimulate the proliferation of duct cells but not the β-cells, and perhaps enhance the differentiation of islet cells (16). It appears that INS-1 cells share some characteristics of β-cell precursors in this respect.

To our surprise, HGF did not have any effect on the proliferation of INS-1 cells. This negative result is likely not due to the use of human recombinant growth factor on rat cells because hHGF has been shown to be fully active on rodent cells (40). HGF has previously been shown to be mitogenic for human fetal (13) and adult (41) β-cells. The data concerning localization of the HGF-receptor (encoded by c-*met*) has been conflicting. While it was found to be particularly high in ducts and β-cells of the human pancreas (13), another study found it only in the ductal epithelium (14). Also in the adult rat, HGF receptors are found in ducts but not in β-cells (S. Bonner-Weir, personal communication). Whereas fetal human β-cells do proliferate in response to HGF (13, 42), it appears that HGF does not induce the replication of adult human β-cells, but instead the proliferation of duct cells (43). Taken together, this suggests that HGF is mainly involved in the stimulation of duct cells, and possibly β-cell progenitors but not the mature β-cells. In this respect, the INS-1 cells represent a more mature phenotype.

Using the INS-1 cell line we were able to identify growth-inhibitory factors that are possibly involved in β-cell differentiation. From these nicotinamide has previously been shown to act as an inducer of endocrine differentiation in fetal human pancreatic cells (44), and to increase islet neogenesis in models of pancreas regeneration (45). Sodium butyrate was another potent inhibitor of INS-1 cell DNA synthesis, which is in line with its well-known activity as a differentiation factor in RIN cells (46). Combining nicotinamide and sodium butyrate led to potentiated differentiative effects with a 4-fold increase in insulin content and decrease of proliferation down to 30% from control.

1,25-$(OH)_2D_3$ induced differentiation of INS-1 cells, indicated by decreasing cell replication and increasing cellular insulin content. 1,25-$(OH)_2D_3$ also stimulated the INS-1 cells to form islet-like clusters instead of growing as a monolayer. These observations are in agreement with previous findings showing that vitamin D analogs act as growth inhibitors on pancreatic adenocarcinoma cell lines (47) and also on RIN cells (48).

Activin A has previously been reported to disrupt epithelial lobulation in the developing mouse pancreas (49). It has also been shown to induce neurite-like processes from exocrine AR42J cells in culture (20). When exposed to activin A together with betacellulin or HGF, these cells are transformed into insulin-secreting cells (20). In accordance with this, we found that activin A was a potent inhibitor of INS-1 cell growth with a concomitant increase in insulin content. These results underline the role of activin A in β-cell differentiation.

We conclude that the INS-1 cell line is a suitable model for the study of β-cell growth and differentiation. It responds to several growth factors similar to primary rat islets. In addi-

tion, we have identified betacellulin as a novel possible mitogen for insulin-producing cells. It appears that the action of betacellulin is not entirely mediated through the known *erb*B receptor tyrosine kinases. We also found 1,25-dihydroxyvitamin D_3, nicotinamide and sodium butyrate to be potent factors for the induction of the genes responsible for β-cell differentiation.

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