BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 851–855 (1997)
ARTICLE NO. RC977399

Rat Insulinoma-Derived Pancreatic β-cells Express a Functional Leptin Receptor That Mediates a Proliferative Response

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Received August 25, 1997

In addition to its interaction at hypothalamic sites to affect feeding and energy expenditure, leptin has been shown to exhibit a proliferative response in erythropoietic cells. The functional leptin receptor is also present in pancreatic islets and we now demonstrate that a commonly used clonal insulin secreting β-cell line, RINm5F, expresses high levels of the Ob-Rb mRNA. Leptin causes an increase in tyrosine phosphorylation of a number of intracellular proteins and a dose related (10nM–200nM) increase in expression of the immediate-early gene, c-fos. This precedes a leptin induced proliferative response in serum-deprived RINm5F cells, which suggests that leptin might be involved in the complex regulation of proliferation of the pancreatic β-cell.

The obese (*ob*) gene product, leptin, is a cytokine belonging to the long chain helical cytokine family that includes for example IL-6, IL-1 and granulocyte colony-stimulating factor (1). Leptin, secreted exclusively from fat cells, plays a major role in neuroendocrine regulation of the body's fat cell mass and energy homeostasis (2). Lack of functional leptin or resistance to its action causes obesity, accompanied by disorders of insulin secretion and hyperglycaemia in several rodent phenotypes (3). The leptin receptor (Ob-R) is expressed as multiple spliced isoforms that possess a common extracellular domain but differ in the length of their cytoplasmic domains (4,5). The Ob-Rb isoform has a long cytoplasmic region, is highly expressed in the hypothalamus and interacts with the Jak/STAT pathway (6).

In addition to hypothalamus, many other tissues express Ob-R, in particular receptor isoforms with short cytoplasmic domains. Current views hold that most of the Ob-R short isoforms are incapable of mediating a leptin signal. In *db*/*db* mice, the cytoplasmic region of the Ob-Rb is truncated due to a mutation resulting in an alternative splice donor site (4). The *db*/*db* mice manifest impaired insulin secretion, diabetes and changes in pancreatic islet morphology. Depending on the background on which the *db* gene is expressed, these mice often have a reduced number of β-cells and atrophic changes in their islets (7-10). Many ligands that activate Jak/STAT signalling pathways affect growth, proliferation and differentiation in a variety of cell types (10-12). These considerations have prompted us to examine if signalling through Ob-Rb is involved in mediating proliferative responses in insulin-secreting cells.

The clonal insulin-secreting RINm5F cell line, derived from radiation-induced rat insulinoma (13), is a commonly used insulin-secreting cell line for studies of pancreatic islet cell biology, including islet growth and apoptosis (14). In the present study, we demonstrate that RINm5F cells express high levels of the full length Ob-Rb mRNA and that leptin induces tyrosine phosphorylation of several proteins leading to a marked increase in expression of the immediate-early gene, c-fos. This precedes a cytokine-like proliferative response in these cells as measured by increased DNA synthesis.

MATERIALS AND METHODS

Cell culture. RINm5F cells (ATCC; Rockville, USA) were maintained in culture in RPMI 1640 medium supplemented with foetal bovine serum (FBS) (10% v/v), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2/air cabinet. Cells were passaged once a week and cells from the passage numbers between 30 and 45 were used for the proliferation assay. Culture components were purchased from Life Technologies (Gibco/BRL).

Cell proliferation assay. Cell proliferation was studied by measuring [methyl-^{3}H]-thymidine (Amersham, UK) incorporation into DNA. RINm5F cells were then incubated in serum-free RPMI 1640 medium, for 24 hours. [^{3}H]-thymidine (0.5 μCi/ml/well) was then

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0006-291X/97 $25.00

added in the presence of recombinant mouse leptin (Amgen Inc., USA), 10% FBS or in RPMI 1640 without supplement. After 24 hours labelling with [^{3}H]-thymidine, the incubation medium was removed and the cells washed twice. Cells were then disrupted with 1% SDS, precipitated with ice-cold 10% trichloroacetic acid (TCA) and pelleted by centrifugation. The radioactivity incorporated was measured by scintillation counting after addition of Unisolve. Radioactivity is expressed as cpm per well. In each experiment 2-5 measurements were performed for each conditions and data are presented as mean± standard error of the mean (S.E.M.).

Leptin-receptor mRNA and c-fos mRNA expression. Total RNA (~40μg) was isolated using RNaid plus kit (BIO 101, Inc. USA) and treated immediately with DNase I (Gibco/BRL, UK), and cDNA synthesized with Superscript II reverse transcriptase (RT) (Gibco/BRL, UK) and oligo $(dT)_{18}$. Primers used for standard PCR amplification of the common extracellular domain of Ob-R (465bp; 275-740; GenBank U60151) were, 5′-GGAATGAGCAAGGTCAAAAC-2′ (sense), 5′-GTGACTTCCATACGCAAACC-3′ (antisense), and for the Ob-Rb isoform (399bp; 2761-3160; GenBank U60151), 5′-AGTGTCGATACAGCTTGG-3′ (sense), 5′-CTGAGAATGAAAGTTGTGG-3′ (antisense). PCR products were then cloned into a pCR-TRAP cloning system (GeneHunter Corp., USA) and their identity confirmed by sequencing using ThermoSequenase terminator cycle sequencing kit (Amersham Life Sciences, UK). The ratio of long form Ob-Rb transcript to that of the total common Ob-R transcript was determined using RNase protection assay. Here, riboprobes (cRNA) were labelled with digoxygenin-UTP (Boehringer Mannheim, Germany) as directed by the manufacturer. Hybridization was performed in 80% formamide buffer overnight at 45°C, then RNA was digested with a cocktail of RNaseT1 and RNaseA. Proteinase K was then added and the protected RNA extracted with phenol:chloroform (1:1) and ethanol precipitated. Protected fragments were resolved on 1.5% agarose gel and then transferred to Hybond N$^+$ membranes (Amersham, UK) for detection. A polyclonal antibody conjugate to alkaline phosphatase (Boehringer Mannheim, Germany) was used to detect digoxygenin-labelled hybrids together with the chemiluminescent substrate CDP-Star (Tropix, USA). RINm5F cells were pre-incubated in serum-free RPMI-1640 for 24 hours and then exposed to leptin (Peprotech, UK) in the same medium for 30 minutes. A digoxygenin-labelled rat c-fos cDNA probe was used in standard northern blot hybridization assay. The c-fos cDNA was PCR amplified from RINm5F cells treated with Interleukin 1-β for 30 minutes using sense primer, 5′-TGCAGCTCCCACCAGTGTCTAC-3′, and antisense primer, 5′-AAGGTAAGGGAAGCCTAAGAGG-3′. This was followed by cloning and sequencing of the cDNA as described above. Loading of RNA was quantitated by scanning laser densitometer analysis of ethidium bromide stained 28S and 18S ribosomal RNA bands in agarose gels.

Immunoblot analysis. Flasks of RINm5F, pre-incubated in serum-free medium, were treated with 100nM leptin (Peprotech, UK) for the indicated time and extracted by addition of boiling 2% SDS, 10mM Tris, pH 7.4. Samples were then spun at 13000rpm for 5 minutes and aliquots removed for protein assay (Sigma, UK) and western blot analysis. Approximately 25 μg of protein was boiled for 3 minutes in a 2× gel loading buffer (4% SDS, 100mM Tris, pH 6.8, 100mM β-mercaptoethanol) and the protein resolved on 8% SDS-PAGE. Proteins were transferred to PVDF (Millipore, UK) membranes by electrotransfer. The membrane was blocked in 2.5% BSA, 10mM Tris, pH 7.4, 100mM NaCl, 0.1% Tween 20 and tyrosine phosphorylation detected by RC20H antibody fragment (Transduction Labs., USA). Bands were visualized using an ECL kit (Amersham, UK) according to manufacturers guidelines.

RESULTS

The effect of leptin on RINm5F cell proliferation was determined by measuring changes in the rate of DNA synthesis. Leptin added to cells in the absence of FBS increased DNA synthesis as measured by [^{3}H]-thymidine incorporation. At a concentration of 10nM leptin, a small increase of proliferation was observed which did not reach statistical significance. Significant stimulation occurred at concentrations of 100 and 200nM (p=0.018, and 0.011 respectively, n=4). The increase in thymidine incorporation stimulated by 200nM leptin was 24-119% (median 49%) over that obtained in controls i.e. in absence of FBS and no other additions (Fig.1B). For comparison, the increase in thymidine

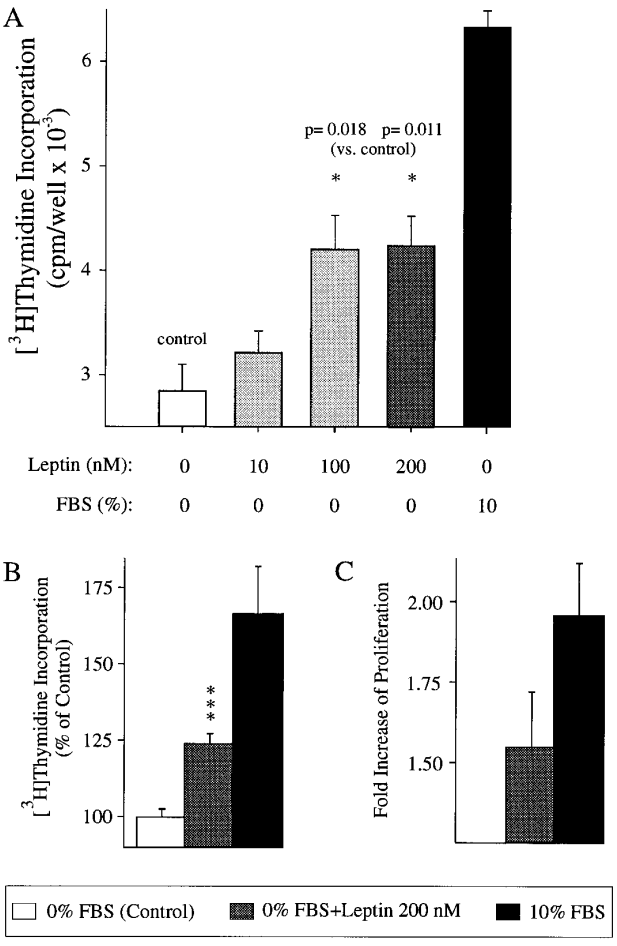

FIG. 1. Stimulation of DNA synthesis in RINm5F cells by leptin, as described under Materials and Methods. **A.** Shows means of duplicate observations ±S.E.M. values of thymidine incorporation stimulated by different concentrations of leptin and 10% FBS. **B.** Data from a separate experiment are expressed as percentage of basal proliferation of control. Values indicate [^{3}H]-thymidine uptake measurements in the absence of FBS, in the presence of 200 nM leptin and in the presence of 10% FBS. Results are mean±S.E.M. values of determinations from five wells. **C.** Results are expressed as -fold increase of proliferation where the proliferation of serum-deprived cells were normalized to 1. Data are mean ±S.E.M. (n=5). Statistical significance was determined by unpaired Student's t test. *, p=0.018 (for 100 nM leptin) and 0.011 (for 200 nM leptin); ***, p=0.0011 all compared to the control.

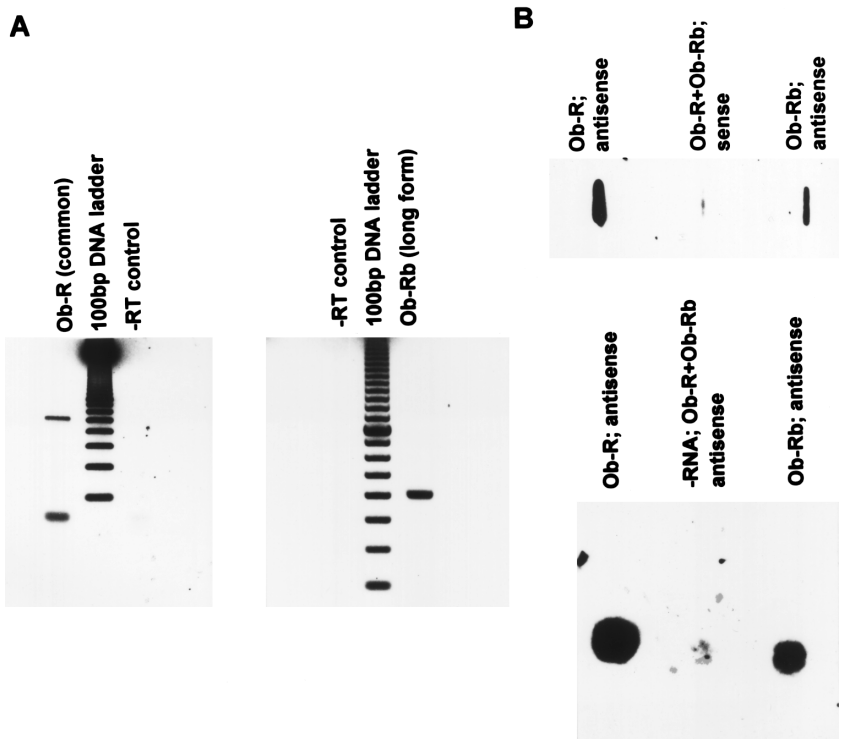

FIG. 2. Ob-R and Ob-Rb mRNA expression in RINm5F cells. **A.** RT-PCR detection of the leptin receptor mRNA in RINm5F cells. Ethidium bromide staining of agarose gels detecting the common Ob-R and the long form Ob-Rb mRNA. The PCR products were cloned and sequenced to confirm their identity. **B.** RNase protection assay using cRNA probes specific for either the common extracellular domain of Ob-R or the long cytoplasmic domain of the Ob-Rb were used in hybridization with total RNA from RINm5F cells. Top panel: specificity of the cRNA probes and capacity of the endonuclease digestion system is tested (samples spotted on membranes). Lower panel: protected fragments fractionated in a 1.5% agarose gels together with -RNA control. A 18S cRNA probe was used to correct for loading differences (data no shown).

incorporation stimulated by 10% FBS was 34-122% (median 117%) over basal. In five experiments, the increase in proliferation of RINm5F cells induced by 200nM leptin varied between 1.24- to 2.19-fold (1.5±0.17-fold, mean±S.E.M., n=5), as compared to proliferation in the absence of both leptin and FBS (Fig. 1C). Under similar conditions, the increase in proliferation by 10% FBS ranged from 1.34- to 2.22-fold (1.9±0.16-fold, n=5) (Fig. 1C). Leptin (200nM) did not induce proliferation in Swiss 3T3 cells (data not shown).

We studied the expression of the common extracellular domain of Ob-R, and the long cytoplasmic domain of the Ob-Rb in RINm5F cells. The RNA was probed for the expression of the long Ob-Rb transcript isoform and the common extracellular domain of the Ob-R transcript. Expression of Ob-Rb mRNA was readily detected by both RT-PCR (Fig. 2A) and RNase protection assay (Fig. 2B), however the transcript of the shorter isoforms predominate. All PCR products and probes were sequenced to confirm identity and found to share complete nucleotide identity to the reported rat Ob-R cDNA (GenBank U60151). The relative percentage fraction of the Ob-Rb transcript to that of the total Ob-R extracellular domain transcript was found to be 24±3.8% (ratio; 0.24±0.04, n=3).

Leptin mediated tyrosine phosphorylation of intracellular protein and the response of the immediate-early gene c-fos were studied as a criterion for functional leptin signalling. We found a time-dependent increase in tyrosine phosphorylation of a number of proteins (Fig 3A). These included increased phosphorylation of bands present in the untreated control (pp130 and pp60) and the appearance of new bands (pp90, pp85 and pp42). This increase was apparent within 2 minutes of exposure, peaked at 5 minutes and fell back to basal within 10 minutes. Next, a specific functional nuclear response was observed by northern blot analysis of c-fos mRNA expression. RINm5F cells incubated with leptin for 30 minutes showed a dose-related increase in c-fos transcript levels at 10nM that became significant at 100nM and was marked (16 fold increase) at 200nM (Fig. 3B).

DISCUSSION

We have previously shown that mouse and rat pancreatic islets express the functional leptin receptor (Ob-

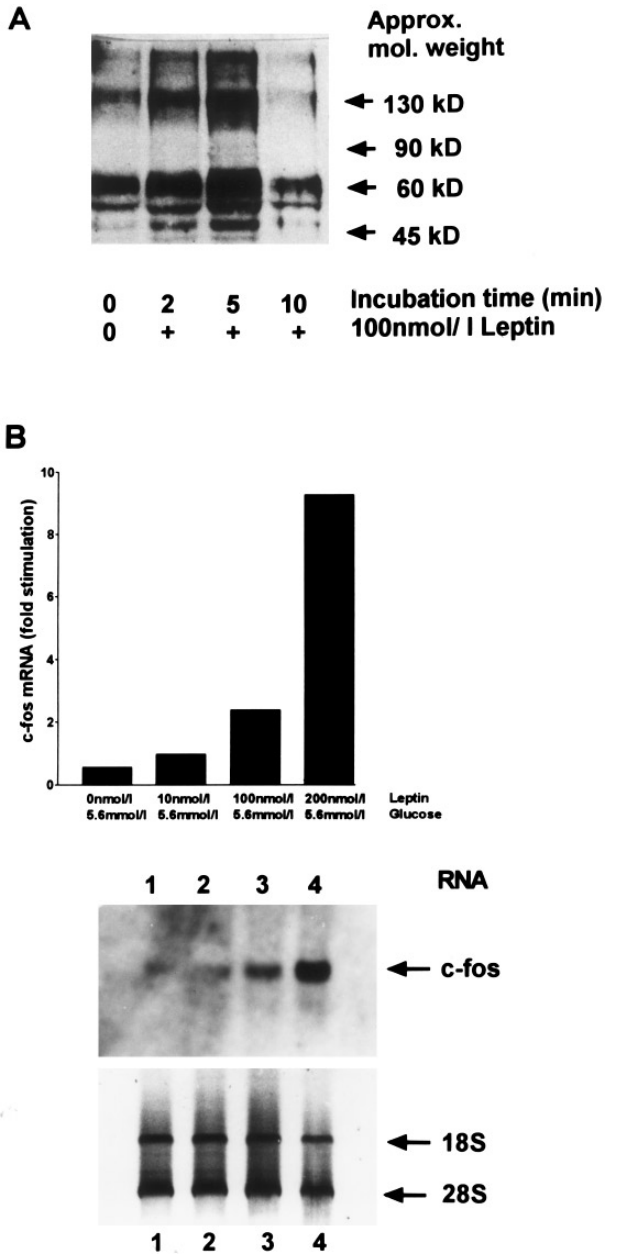

FIG. 3. Leptin induced tyrosine phosphorylation and c-fos mRNA expression in RINm5F cells. **A.** RINm5F cells pre-incubated in serum-free medium were exposed to 100nM leptin for 2, 5 and 10 minutes. Cellular protein was fractionated in 8% SDS-PAGE, blotted and then probed with HRP-conjugated antibody to phosphorylated tyrosine moieties. **B.** Northern blot hybridization of total RNA from serum-deprived RINm5F cells, that were exposed for 30 minutes to basal (lane 1), leptin at 10nM (lane 2), 100nM (lane 3) or 200nM (lane 4).

Rb) and that leptin mediates a dose dependent inhibition of insulin secretion in these systems (15, 16). We now demonstrate that Ob-Rb mRNA is readily detected in an immature β-cell model, the rat-insulinoma derived RINm5F cell, however the shorter transcript isoforms predominate. Here, expression level of Ob-Rb mRNA to that of the total Ob-R mRNA was abundant or 24$\pm$3.8%, as measured by the RNase protection assay. This is the highest relative abundance of the full length Ob-Rb transcript in peripheral tissues or related cell lines, reported to date (see ref. 17 for comparison).

We show that leptin increases tyrosine phosphorylation of a number of proteins with maximal activation observed after 5 minutes. Although the identity of the bands has not been confirmed in this study their approximate sizes correlate well to several proteins which have been implicated in leptin receptor signalling by other workers (6, 17), thus, pp130 and pp85-90 may represent the Jak/STAT family. It is also possible that pp42 and pp45 may represent MAP kinase family members. The identity of the pp60 protein which undergoes a marked increase in tyrosine phosphorylation is unknown, however, there is evidence that this protein is also appreciably activated by leptin in isolated islets of Langerhans of *ob*/*ob* mice after leptin treatment (18).

In this study, leptin is found to cause a concentration-dependent increase in [^{3}H]-thymidine incorporation into serum-deprived RINm5F cells. Proliferation of cells induced by leptin is not a widely occurring phenomenon and has so far been reported only in hematopoietic cell types (19, 20). We believe that the proliferative response to leptin is mediated through Ob-Rb. The significance of the proliferative response to leptin in this tumour cell line needs to be carefully considered. The requirement for somewhat high concentrations of leptin for demonstrating proliferative response could be explained by the potential ability of leptin to down-regulate its own receptor (21). Moreover, leptin is normally present in serum, thus the cells during cultivation have been exposed to leptin continuously. We have observed that RINm5F cells which are not pre-incubated in the absence of FBS show no significant changes in either crude cellular tyrosine phosphorylation or induction of c-fos mRNA at concentrations of 100nM leptin (data not shown). To date, receptor binding assays (22) and identification of Ob-Rb mRNA in insulin-secreting cells (15, 16 and the present study), the effects of leptin on insulin secretion (15) and insulin level (23), provide substantial evidence for the presence of sufficient functional quantities of Ob-Rb in β-cells. This makes these cells a direct target for leptin action (15).

The fact that leptin increases proliferation of RINm5F cells suggests that this cytokine may have a positive regulatory function on β-cell growth and proliferation. However, the effect of leptin on β-cells may be complex depending on the context in which stimulation by the hormone takes place and the stages in the development of these cells. Our previous work was the first to demonstrate an inhibitory effect of leptin on glucose-stimulated insulin secretion in mature *ob*/*ob* islets (15). A proliferative effect on β-cells might result from chronic stimulation by leptin and may be relevant at

early stages of their development and/or in conditions associated with β-cell hyperplasia. An association between normoglycemic obesity and β-cell hyperplasia has been described by Ogilvi as early as in 1933 (24) and has since been documented in animals and humans (25-27). Such an association between islet hypertrophy and obesity could be explained by a chronic leptin-mediated stimulation on the pancreatic β-cell.

ACKNOWLEDGMENTS

Financial support was obtained from Åke Wiberg stiftelse, The Swedish Society of Medicine and funds of the Karolinska Institute. M.S.I. has a position at the Swedish medical Research Council. N. Morton is supported by a research studentship from SCRAS. We thank David Hislop and Amber Kanu for expert technical assistance and Michael A. Cawthorne for critical discussion of the manuscript.

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