Endocrine (1995) 3, 653-660

Evidence for a role of insulin in hepatocytic differentiation of human hepatoma BC1 cells

Sukmi Kang-Park[1], Jacqueline Capeau[1], Annie Munier[1], Martine Caron[1], Denise Glaise[2], Christiane Guguen-Guillouzo[2], Gisèle Cherqui[1] & Olivier Lascols[1]

[1]*Laboratoire de biologie cellulaire, INSERM U402, Faculté de Médecine Saint-Antoine, 75571 Paris Cedex 12, France and* [2]*INSERM U49, Hôpital de Ponchaillou, 35033 Rennes, France*

To examine the effect of insulin on hepatocytic differentiation, we took advantage of the properties of the newly established human hepatoma BC1 cell line to maintain quiescence after confluency and to progressively acquire in culture (3 weeks after confluency) an hepatocytic phenotype, as assessed by expression of specific hepatic genes (Le Jossic *et al.*, 1995). In BC1 cells cultured in the presence of insulin (1 μM), expression of albumin and transferrin mRNA and protein occurs earlier than in cells cultured in its absence (1 week *vs* 2 weeks). Moreover, at any time considered, the level of the two hepatic markers was higher (2- to 3-fold) in the former than in untreated cells. The beneficial effect of insulin on hepatocytic differentiation of BC1 cells was paralleled by: i) modest increases in insulin receptor (IR) mRNA level and IR binding activity, and ii) a 6-fold increase in sensitivity to insulin for stimulation of glycogenesis. These results provide the first evidence for insulin's ability to exert a positive effect on hepatocytic differentiation. The beneficial effect of insulin probably results both from increased IR expression and binding activity and from alteration at post-receptor levels.

Keywords: human hepatoma; insulin; hepatocytic differentiation; insulin receptor isoform; albumin; transferrin

Introduction

It is well known that insulin acts as a metabolic and/or mitogenic factor in various cell types, including hepatocytes and hepatoma cells (Hoffman *et al.*, 1989; Thompson *et al.*, 1991; Koontz & Iwahashi, 1981). Otherwise, as concerns its ability to promote differentiation, several groups reported that insulin accelerated adipocyte differentiation (Gaben-Cogneville *et al.*, 1988; Hauner, 1990; Sadowski *et al.*, 1992). This process was found to be associated with altered insulin receptor expression (Mosthaf *et al.*, 1990; Smith *et al.*, 1988) or function (Rubin *et al.*, 1978) or with increased insulin signaling (Saad *et al.*, 1994; Rice *et al.*, 1992). By contrast, even though insulin is often used to maintain hepatocytes in primary culture (Guguen-Guillouzo *et al.*, 1986; Hoffman *et al.*, 1989), little is known about the effect of insulin on hepatocyte differentiation. A major aspect of this process is the increased expression and secretion of numerous plasma proteins, the most abundant of which is albumin. Discrepant results were previously presented concerning the effect of insulin on albumin expression and/or synthesis. At first, *in vivo* studies demonstrated that insulin increased the amount of albumin mRNA as well as the synthesis and secretion of the protein in rat liver (Jefferson *et al.*, 1983). Subsequent *in vitro* studies showed that removal of insulin from the medium of rat hepatocytes maintained in primary culture decreased albumin gene transcription and that conversely, addition of insulin to the medium reversed this defect (Lloyd *et al.*, 1987; Caron, 1990). However, these findings failed to be corroborated by other work which demonstrated that, in a cultured rat hepatoma cell line (H4IIE), insulin decreased the cellular concentration of albumin mRNA (Straus & Takemoto, 1987) as well as the transcription of its gene (Messina, 1992). Therefore, the question of whether insulin exerts a beneficial effect on hepatocyte differentiation and, if so, by which mechanisms, remains to be addressed.

In the present work we studied a newly established human hepatoma cell line BC1 (Le Jossic *et al.*, 1995) which was shown to acquire an hepatocytic phenotype when cultured in the presence of fetal calf serum (FCS), hydrocortisone and insulin. We took advantage of this property to examine the specific effect of insulin on the differentiation of BC1 cells. To this end, i) we compared BC1 cells maintained throughout the culture time (1 to 4 weeks) either in the absence or in the presence of insulin for expression of albumin and transferrin, two secreted proteins which are known as specific hepatic markers (Ng *et al.*, 1993; De Juan *et al.*, 1992); ii) we examined BC1 cells cultured in the presence of insulin during 1 or 4 weeks for insulin receptor expression and functions and their response to insulin for stimulation of glycogenesis, a major hepatic metabolic function. Our results provide the first evidence that insulin not only accelerated hepatocytic differentiation of BC1 hepatoma cells but also markedly amplified this process through a mechanism which is likely to take place both at receptor and post-receptor levels.

Results

Expression of hepatic markers in BC1 hepatoma cells in the absence of insulin

To investigate whether BC1 and B9 hepatoma cells presented hepatic specific markers, we examined the expression of the two differentiation markers, albumin and transferrin. After 1 week in culture when BC1 and B9 cells have reached confluency, albumin and transferrin mRNAs were expressed at very low levels in both cell lines (Figure 1). By contrast, after 4 weeks the level of the mRNA of the 2 hepatic markers was markedly increased in BC1 cells but remained unmodified in B9 cells. This result indicated that B9 and BC1 cells, which were derived from the same hepatocarcinoma, express distinct phenotypes. Indeed, only BC1 cells expressed specific markers of hepatocyte phenotype at a level which, however, was lower than that observed in isolated human hepatocytes and well-differentiated (1-week) HepG2 cells (Figure 1).

Effect of long-term insulin treatment on albumin and transferrin expression and secretion in BC1 cells

We then tested the effect of insulin on the hepatocytic differentiation of BC1 cells. To this end, we measured albumin and transferrin mRNA levels in cells cultured without or with insulin (1 μM) for 1 to 4 weeks (Figure 2A). In the absence of insulin, the mRNA levels of albumin and

Correspondence: Dr O. Lascols
Received 2 March 1995; accepted 19 June 1995

transferrin progressively increased during culture up to 3 and 6-fold, respectively (Figure 2A). However, insulin induced an additional increase in both albumin and transferrin mRNA levels. This effect was seen even after the first week when albumin and transferrin mRNA levels were increased by 2.2- and 3.3-fold, respectively as compared with cells cultured in the absence of insulin (Figure 2A). The level of the response was distinct for the two mRNAs. Albumin mRNA level was raised up to a maximum of 6.7-fold after 4 weeks and the transferrin mRNA level was maximally increased by 17-fold after 4 weeks as compared with untreated control cells. Under this condition the level of β-actin mRNA remained unmodified (see Figure 5). Therefore, in the presence of insulin, the expression of specific hepatic markers in BC1 cells occurred earlier and was higher than in its absence, indicating the ability of this hormone to accelerate and amplify the differentiation process. By contrast, cell growth was not significantly modified when the cells were cultured in the presence of FCS and insulin as compared to FCS alone (data not shown).

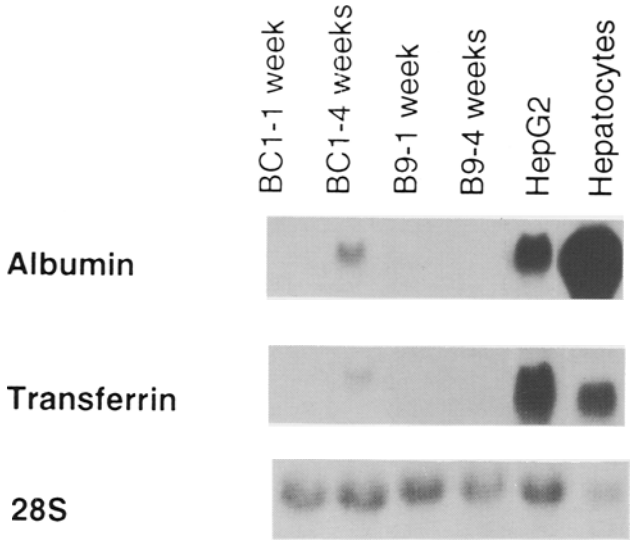

Figure 1 Northern blot analysis of albumin and transferrin mRNA expression. Total cellular RNA was prepared from confluent (1-week) and 3 week post-confluent (4-weeks) BC1 and B9 human hepatoma cells, confluent HepG2 human hepatoma cells and isolated human hepatocytes and analysed by Northern blot (15 μg/lane) using radiolabeled human cDNA probes for albumin and transferrin as described in Materials and methods. Filters were subsequently hybridized with a 28 S rRNA probe labeled with T4 kinase and [γ-^{32}P]ATP. Autoradiograms of a representative experiment are shown

To determine whether the increased levels of albumin and transferrin mRNAs resulted in increased protein expression, the secretion of albumin and transferrin by BC1 cells was evaluated throughout culture. BC1 cells were cultured in the absence or presence of insulin as described above. The medium of the last 24 h was collected at various stages of culture and analysed by Western blotting for the presence of albumin and transferrin (Figure 2B). When cells were grown in insulin-free medium, the secretion of albumin and transferrin progressively increased to reach after 4 weeks a level which was respectively 3.4- and 5.3-fold higher than that measured after 1 week. The presence of insulin (1 μM) in the culture medium promoted a further increase to reach a 7.2- and 11-fold stimulation of albumin and transferrin secretion in 4-week cells as compared to 1-week cultured cells. These values are in good agreement with those observed for mRNA levels and confirmed that insulin facilitated the acquisition by

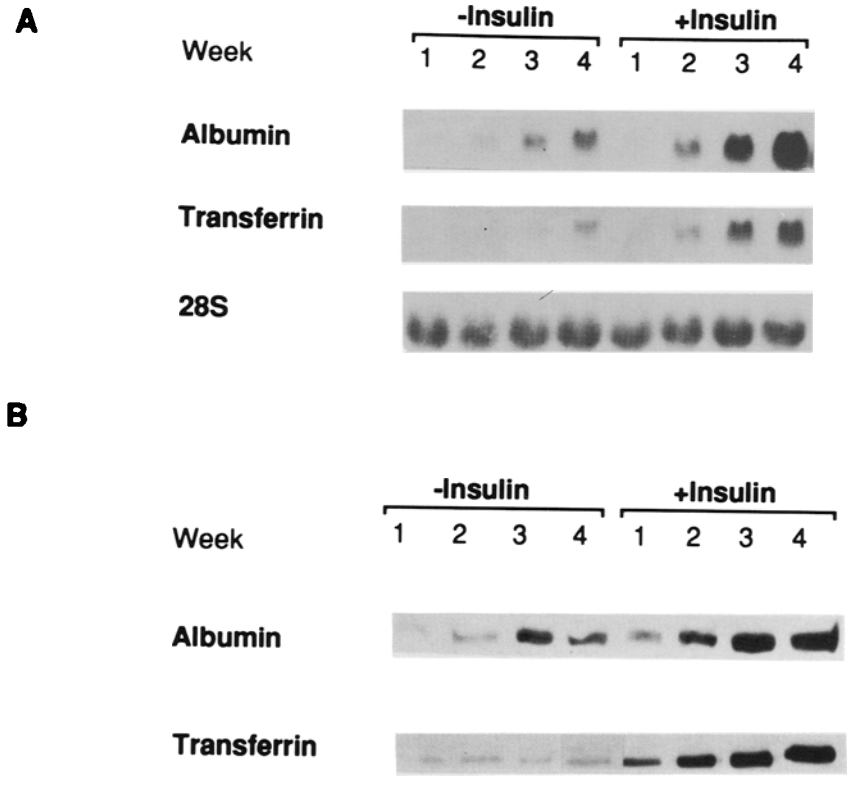

Figure 2 Effect of long-term treatment with insulin on albumin and transferrin mRNA levels and in secretion by BC1 cells. Cells were cultured in the absence or presence of insulin (1 μM) for 1 to 4 weeks. The medium was changed every 2 days. (**A**) mRNA levels. Northern blot analysis of total RNA (15 μg/lane) with ^{32}P-labeled cDNA probes for albumin or transferrin, or a oligonucleotide probe for 28S rRNA as described in Materials and methods. (**B**) Protein secretion. Cells in FCS were cultured in the absence (−) or presence (+) of insulin (1 μM) for the indicated times after seeding. Then cells were incubated in 4 ml of medium MEM + M199, for the following 24 h. Media were collected and proteins (45 μl/lane) were separated by SDS/10% PAGE. Western blot analysis was performed with specific anti-human albumin or anti-human transferrin antibodies and a secondary peroxidase-conjugated antibody as described in Materials and methods

BC1 cells of an hepatocytic phenotype with secretion of specific proteins.

In view of the above results we sought to examine whether the long-term effect of insulin was: (i) independent of a possible effect of FCS which was maintained at a 10% concentration throughout culture and (ii) actually mediated by insulin receptors. To address the first point, BC1 cells were cultured in the absence of insulin (+ 10% FCS) for 3 weeks and then treated with insulin (1 μM) for 6 days, in the absence or presence of 10% FCS. Northern blot analysis

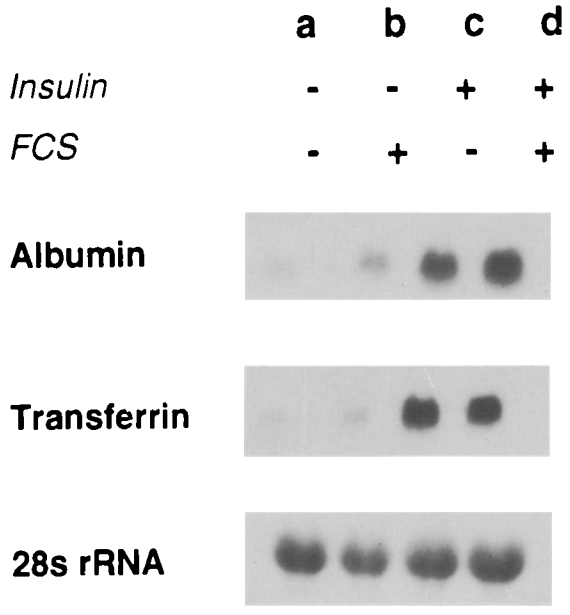

Figure 3 Comparative effects of FCS and insulin on albumin and transferrin mRNA levels. BC1 cells were cultured in insulin-free medium for 3 weeks. Chronic insulin treatment (1 μM) was performed (lanes c, d) or not (lanes a, b) for the 6 following days, in the absence (lanes a, c) or presence (lanes b, d) of 10% FCS. Medium was changed every day. Total cellular RNA was extracted and Northern blot analysis (15 μg/lane) was carried out with specific cDNA probes or a oligonucleotide probe as described in Materials and methods. Autoradiograms of a representative experiment are shown

(Figure 3) clearly showed that FCS was able to increase slightly the expression of albumin and transferrin. However, insulin had a far higher effect which was not modified in the presence of FCS. These data clearly established an intrinsic effect of insulin on hepatocytic differentiation of BC1 cells which was independent of FCS. To address the second point, we investigated the effect of 10^{-8} M insulin on the level of albumin and transferrin mRNAs and compared this effect to that elicited by 10^{-8} M IGF-I. We found (data not shown) that both agonists were able to increase the expression of these 2 markers at 10^{-8} M. We then determined the ability of insulin to cross-react with IGF-I receptors by determining the specific binding of [^{125}I]IGF-I to BC1 cells at a tracer concentration (3.4×10^{-11} M) in the absence or presence of graded concentrations (2×10^{-10} to 2×10^{-5} M) of either unlabeled IGF-I or insulin. As shown in Figure 4A, native IGF-I inhibited specific [^{125}I]IGF-I binding in a concentration-dependent manner, with an ED_{50} of 1.9×10^{-9} M. In contrast, at a concentration as high as 5×10^{-8} M, insulin proved unable to inhibit [^{125}I]IGF-I binding to IGF-I receptors, indicating a very poor affinity of IGF-I receptors for insulin in BC1 cells. This finding, together with the finding that insulin was able to increase the expression of albumin and transferrin, when used at 10^{-8} M, led us to conclude that insulin is able to signal the hepatocytic differentiation of BC1 cells through its own receptors. However, the fact that insulin was added to 10^{-6} M [in order to compensate for its high degradation rate (86% after 2 days of culture) in BC1 cells], raises the possibility that part of the effect of insulin on hepatocytic differentiation could be mediated by IGF-I receptors.

Effect of long-term insulin treatment on insulin receptor expression and function

We next examined whether hepatocytic differentiation of BC1 cells in the presence of insulin correlated with changes at the insulin receptor level. To this end, we first studied insulin binding and insulin receptor expression. We analysed the binding properties of the IRs present on the plasma membrane of BC1 cells after 1 or 4 weeks of culture in the presence of insulin. In competitive inhibition experiments (Figure 4B), the ED_{50} was slightly decreased at 4 weeks as compared to that measured at 1 week (1.8×10^{-10} M and 3.4×10^{-10} M, respectively). Scatchard analysis of these data indicated that

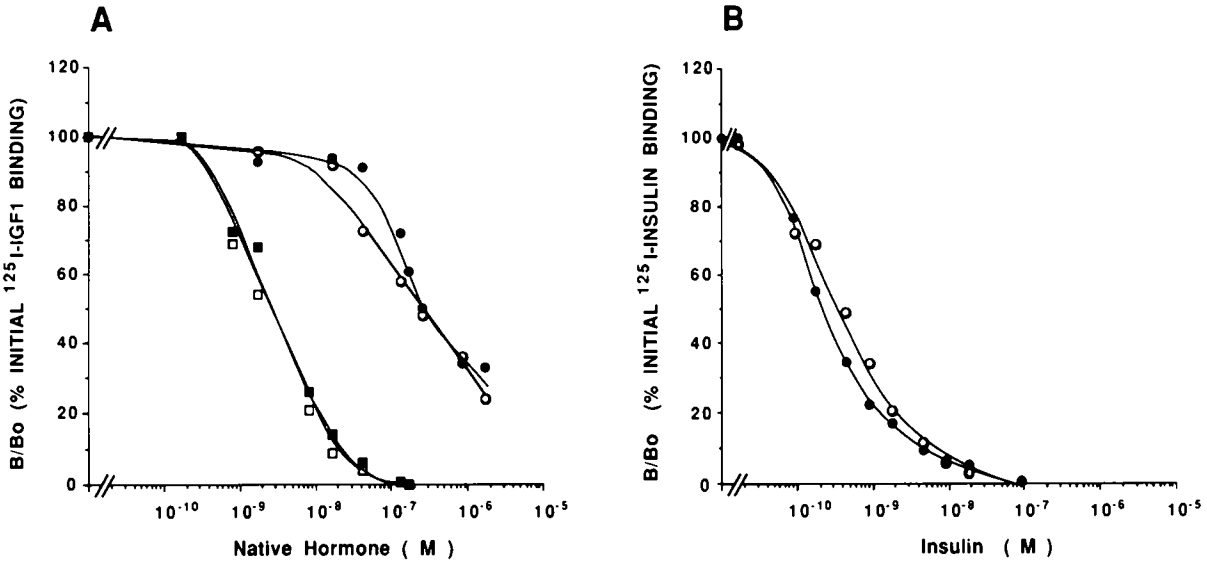

Figure 4 (A) Competition curves of specific [^{125}I]IGF-I binding (A) and [^{125}I]insulin binding (B) to 1-week- and 4-week-insulin-treated BC1 cells by unlabeled IGF-I or insulin. Binding of [^{125}I]IGF-I and [^{125}I]insulin to 1-week (□, ○) and 4-week (■, ●) BC1 cells in the absence or presence of the indicated concentrations of unlabeled IGF-I (□, ■) or insulin (○, ●) was performed as described in Materials and methods. The data represent the mean of 3 to 7 experiments performed in duplicate. The curves were generated with a computational program (RIACALC Lab Manager (LKB-Pharmacia))

this alteration of ED_{50} during differentiation resulted from a 2.5-fold increase in the high affinity binding component ($K_d = 0.9 \times 10^{-10}$ *vs* 2.3×10^{-10} M respectively).

Northern blot analysis (Figure 5A) revealed the presence of two IR specific mRNA transcripts of 10.2-kb and 9.3-kb in BC1 cells which had been incubated for 1 or 4 weeks with insulin. The levels of both transcripts were increased by 1.5-fold in the differentiated 4-week cells as compared to the level measured in 1-week cells. To determine the relative levels of mRNA encoding the A and B isoforms of the IR, total RNA was subjected to reverse transcription (RT) and amplification by PCR. The bands of 446 and 482 corresponded to the PCR products of A (−exon 11) and B (+exon 11) isoforms, respectively (Figure 5B). Ubiquitous Glut1 transporter was used as an RT/PCR internal standard. The level of the IR-A isoform increased in differentiated cells whereas the level of the IR-B isoform, which was the major isoform in these cells, was not modified. Thus the change in the ratio of IR-A to B isoforms throughout the BC1 differentiation process was associated with increased IR affinity for insulin.

We next determined IR tyrosine kinase activity by measuring either IR autophosphorylation or phosphorylation of an exogenous substrate, poly(Glu, Tyr) (PGT). As shown in Figure 6A, the autophosphorylation of the IR 95 kD β subunit measured in WGA-purified fractions containing similar levels of IRs (see legend to Figure 6) was stimulated by insulin to the same extent as 1 week- and 4 week-insulin-treated BC1 cells (lane 2 and 4). When the phosphorylation assay was carried out using PGT as an exogenous substrate, there was no change in the insulin-concentration response curves (Figure 6B). The similar values for the ED_{50} (1.5 and 1.7×10^{-8} M) and maximal responses (144–155% over basal) indicated that the IR presented no modification of the intrinsic tyrosine kinase activity throughout the hepatocyte differentiation process of BC1 cells.

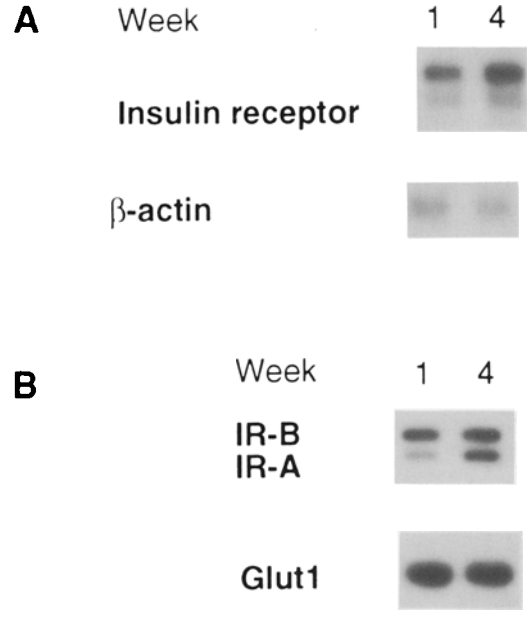

Figure 5 Expression of IR mRNA in BC1 cells. Total cellular RNA was prepared from 1-week and 4-week BC1 cells cultured in the presence of insulin. Poly(A)$^+$ fraction was purified as described in Materials and methods. (**A**) Total IR mRNA level was measured by Northern blot analysis. Each lane contained 2 μg of RNA. Filters were hybridized with a ^{32}P-labeled cDNA probe for the human insulin receptor and subsequently with a β-actin oligonucleotide probe labeled with T4 kinase and [γ-^{32}P]ATP as control. (**B**) PCR amplifications of the two IR isoforms. The two isoforms were separated by electrophoresis in 2% agarose gels. The amplified DNA fragments of A- and B-isoforms were 446 and 482 bp in length, respectively. The Glut 1 was used as a RT/PCR internal standard. Autoradiograms of representative experiments are shown

Effect of long-term insulin treatment on BC1 cell sensitivity to insulin for stimulation of glycogen synthesis

In view of the above results, we next investigated the effect of insulin on glycogenesis, an important hepatic metabolic function. In these experiments, BC1 cells which had been treated for 1 or 4 weeks with insulin were incubated for 1 h with 0.6 μCi/ml [U-^{14}C]glucose in the presence of graded concentrations (0 to 10^{-6} M) of insulin (Figure 7). Insulin increased glycogen synthesis in both cases in a dose-dependent fashion, but the concentration-response curves differed. After 4 weeks of insulin treatment, BC1 cells exhibited a higher sensitivity to insulin for the stimulation of this process as indicated by the 6-fold leftward shift ($ED_{50} = 6.5 \times 10^{-10}$ M) of their concentration-response curve as compared to that measured in cells treated with insulin for 1 week ($ED_{50} = 3.8 \times 10^{-9}$ M).

These results indicated that BC1 cells which have undergone an hepatocytic differentiation in the presence of insulin, displayed an increased sensitivity to insulin for stimulation of glycogen synthesis.

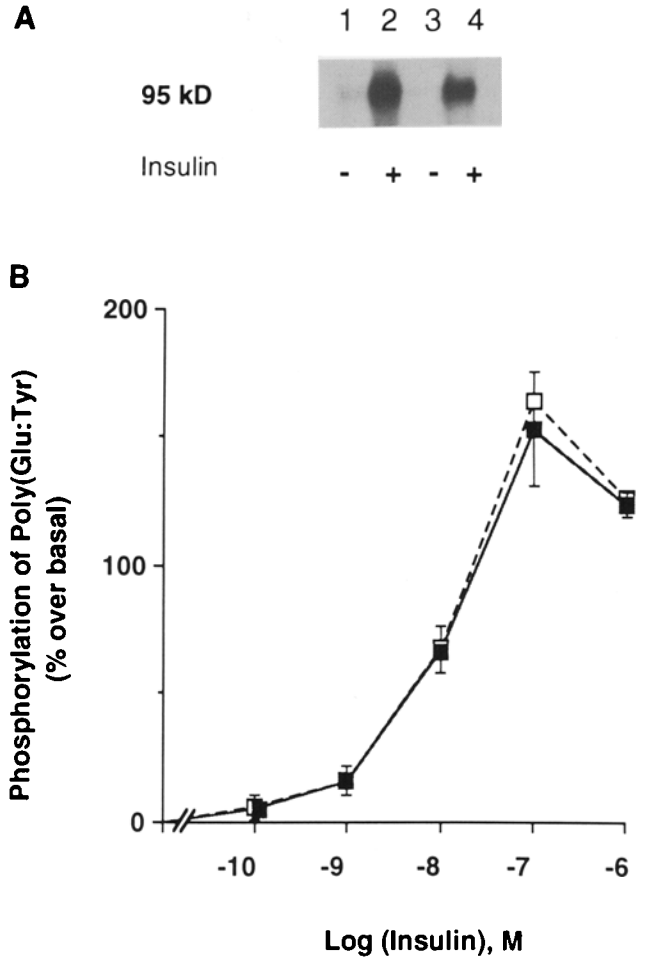

Figure 6 Autophosphorylation and tyrosine kinase activity of the insulin receptor. (**A**) Autoradiogram of insulin-stimulated phosphorylation of a 95 kD protein from 1-week (lanes 1, 2)- and 4-week-insulin-treated (lanes 3, 4) BC1 cells. WGA-purified receptors (2 μg of protein, corresponding to 800 and 760 c.p.m. specific insulin binding for non-differentiated and differentiated cells respectively) were incubated for 40 min at 25°C in the absence (−) or presence (+) of insulin (1 μM). Phosphorylation was carried out for 10 min as described in Materials and methods. (**B**) WGA-purified receptors (1 μg) from 1-week (□)-and 4 week-insulin-treated (■) BC1 cells were incubated for 40 min at 25°C in the presence of the indicated concentrations of insulin. Phosphorylation of PGT (0.7 mg/ml) was then performed for 10 min as indicated in Materials and methods. The basal PGT phosphorylation activity was 6.3 and 4.8 fmol ^{32}P/min/μg protein respectively for 1-week and 4-week cells. Results are expressed as the percent ± SEM over basal of PGT phosphorylation and are the mean of 3 independent experiments

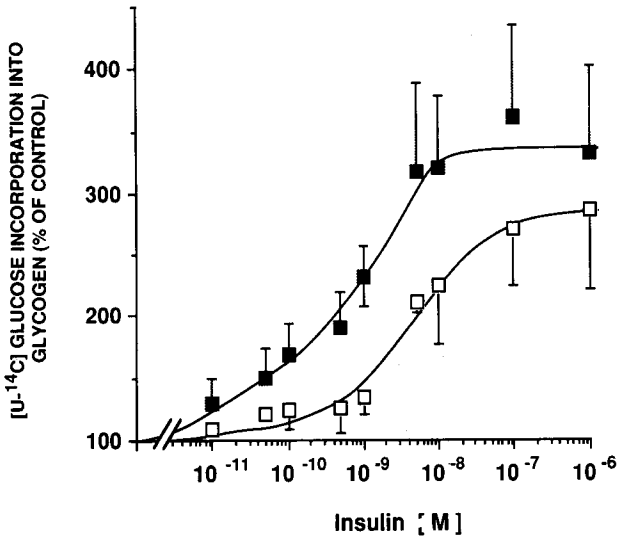

Figure 7 Effect of insulin on [U-^{14}C]glucose incorporation into glycogen in 1-week (□) and 4-week (■) insulin-treated BC1 cells. Cells in 12-well plates were deprived of FCS and insulin, and incubated for 1 h with 0.6 μCi/ml [U-^{14}C]glucose in the presence of the indicated concentrations of insulin. Glycogen extraction was performed as described in Materials and methods. Results are expressed as the percentage value of the corresponding control and are the mean ± SEM of 4 experiments

Discussion

It is generally assumed that hepatocyte proliferation and differentiation are antagonistic processes (De Juan *et al.*, 1992). Indeed, it has been shown that hepatocyte proliferation in culture is accompanied by a diminution of hepatic markers (Bernuau *et al.*, 1988, Guguen *et al.*, 1975). To analyse the effect of insulin on hepatocyte differentiation, Crettaz and Kahn (1983) initially used rat clonal hepatoma cell lines derived from the H35 Reuber hepatoma. However, in these cells, insulin promoted cell proliferation (Koontz & Iwahashi, 1981; Shimizu & Shimizu, 1986) but failed to induce the expression of hepatocyte-specific genes (Straus & Takemoto, 1987; Messina, 1992). Furthermore, the extensively-studied HepG2 human hepatoma cell line does not appear to be adapted for studying hepatocyte differentiation since these cells are growing continuously in culture. In contrast, since the recently described human hepatoma BC1 cell line (Le Jossic *et al.*, 1995) remained quiescent after confluency, it provided us an interesting model to study the effect of insulin on hepatocyte differentiation.

In this work we observed that under appropriate culture conditions (i.e., in the presence of 0.1 μM hydrocortisone and 10% fetal calf serum), human hepatoma BC1 cells initiate an hepatocytic differentiation process as indicated by weak albumin and transferrin expression and secretion taking place during 4 weeks of culture. Of particular interest is the finding that this process can be markedly accelerated and amplified by chronic treatment with insulin (1 μM). This is assessed by the finding that mRNA expression and protein secretion of both albumin and transferrin occurred earlier and were higher in the presence of insulin than in its absence. Several inducers such as laminin (Bissell *et al.*, 1987; Reif *et al.*, 1991) or dimethylsulfoxyde (DMSO) (Chan *et al.*, 1989) in cultured hepatocytes, acidic fibroblast growth factor (aFGF) (Marsden *et al.*, 1992) in rat liver, or dexamethasone (Kosaki & Webster, 1993) in HepG2 cells, have been shown to maintain and/or enhance hepatocyte differentiation. The results presented here provide the first evidence that insulin may also exert a beneficial effect on hepatocyte differentiation. The major role played by insulin compared to other factors in this process is indicated by the absence of hepatocytic differentiation of BC1 cells cultured in the presence of hydrocortisone alone and the low level of differentiation when cultured in the presence of FCS and hydrocortisone. This study shows that insulin is able to mediate hepatocytic differentiation through its own receptors. This is supported by the finding that at 10^{-8} M, a concentration at which insulin did not displace ^{125}I-IGF-I from IGF-I receptors, it is able to increase albumin and transferrin mRNA expression. In addition, since IGF-I is also able to promote increased albumin and transferrin expression through its own receptors, it is possible that, under the conditions of the study (10^{-6} M insulin), part of the effect of insulin could be mediated by IGF-I receptors. Consistent with the results presented here, Accili & Taylor (1991) reported a key role for insulin in inducing the adipocyte differentiation of 3T3-L1 cells, even though part of the effect of the hormone could be mediated by IGF-I receptors (Smith, 1988).

The differentiation of 3T3-L1 cells was accompanied by a 13-fold increase in insulin binding (Gaben-Cogneville *et al.*, 1988) and insulin receptor mRNA which was paralleled by a 9-fold increase in IRS-1 level (Saad *et al.*, 1994). This is in marked contrast with what was observed in differentiated BC1 cells, in which insulin receptor mRNA level was only increased by 1.5-fold. However, in these cells the insulin receptors expressed at the plasma membrane exhibited an increased affinity for insulin, as indicated by a 2-fold leftward shift of the competition curve for insulin binding. This increase in insulin receptor affinity was correlated with an increased expression of IR-A isoform (−exon 11) which was previously shown (Mosthaf *et al.*, 1990; McClain, 1991, Yamaguchi *et al.*, 1993) to display a higher affinity for insulin than the IR-B isoform (+exon 11). Indeed, our results show that, in BC1 cells IR-B isoform was the major isoform as is also the case in normal liver cells (Benecke *et al.*, 1992; Mosthaf *et al.*, 1990) but not in hepatoma cell lines, such as FAO and HepG2 cells, and other human hepatoma cells (Seino & Bell, 1989; Sell *et al.*, 1994). Throughout the differentiation process of BC1 cells, the ratio of IR-A/IR-B isoforms progressively increased since IR-A expression increased whereas that of the IR-B isoform did not change. Since dexamethasone was recently reported to cause a switch in the expression of the IR-A toward IR-B isoform in HepG2 cells (Kosaki & Webster, 1993), the possibility that the presence of hydrocortisone (0.1 μM) in the culture medium together with insulin could be responsible for increased IR-A isoform expression seems unlikely. Otherwise, insulin was reported to acutely stimulate IR-B isoform in rat hepatoma FAO cells (Sell *et al.*, 1994) but expression of the IR-A isoform was increased in muscles from insulin-sensitive as compared in insulin-resistant patients. We show here that a long-term treatment with insulin increased the expression of the IR-A isoform which however remained the minor one. Further studies will be required to define precisely the respective role of acute *vs* long-term treatment with insulin. When IR tyrosine kinase activity was expressed relative to receptor number, we failed to detect any modification throughout differentiation of BC1 cells. This may not be surprising since IR-A and IR-B isoforms have already been reported to display a similar IR tyrosine kinase activity in CHO cells (Yamaguchi *et al.*, 1991).

In addition to changes in insulin binding and IR isoform expression during insulin-induced differentiation, BC1 cells became more sensitive to insulin for glycogen synthesis (Figure 7). Our results indicate that both non-differentiated and differentiated BC1 cells could respond to insulin with different sensitivities, which probably resulted from the observed differences in receptor expression and affinity. Therefore, IRs were already present and functional in non-differentiated BC1 cells. Since the differentiation process took place only after 1 week, we could postulate that the pathway whereby insulin enhances hepatocyte differentiation of BC1 cells involves the participation of some post-receptor steps

that couple insulin receptor activation to insulin-induced BC1 cell differentiation. As a late post-receptor event, implication of hepatic transcription factors, such as HNF-1, HNF-4 and C/EBP in the regulation of liver differentiation (Cereghini *et al.*, 1990; Mueller, 1992; Nagy *et al.*, 1994) has been reported. The presence of HNF-1 and HNF-4 was in fact found in differentiated BC1 cells (Le Jossic *et al.*, 1995). Thus it is possible that insulin activates specific transcription factors involved in the hepatocytic differentiation of BC1 cells. This point remains to be elucidated.

In summary, using the newly characterized human hepatoma BC1 cell line, we provide the first evidence for insulin's ability to accelerate and increase hepatocyte differentiation. We show that the effect of insulin on this process resulted at least in part from the capacity of the hormone to increase IR expression and improve IR function. However, the fact that functional IRs were already present in the non-differentiated state and, moreover, that the IR level and activity were modestly increased in differentiated BC1 cells, leads us to suggest that the beneficial effect of insulin on hepatocytic differentiation of BC1 cells also involves stimulation of the expression and/or activity of IR downstream signaling molecules. The results presented here demonstrate that this human hepatoma BC1 cell line provides a suitable *in vitro* model to study the implication of signaling molecules playing a role in the process of hepatocyte differentiation.

Materials and methods

Materials

Medium 199, minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and *Moloney Murine Leukemia* Virus (Mu-MLV) reverse transcriptase were purchased from Gibco BRL. (3-[^{125}I]-iodotyrosyl^{A14}) insulin (2000 Ci/mmol), (3-[^{125}I]-iodotyrosyl)insulin-like growth factor-I (IGF-I) (2000 Ci/mmol), D-[U-^{14}C]glucose (293 mCi/mmol), [γ-^{32}P]ATP (10 - 30 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), Megaprime DNA labelling system, and ECL western blotting detection reagents were purchased from Amersham. The polyclonal anti-human transferrin antibody was obtained from ICN. The anti-human albumin antibody and the goat anti-IgG-peroxidase-conjugated antibody were purchased from Biosys. RNAzol^{TM}B was obtained from Bioprobe. Bovine insulin (28.6 U/mg), bovine serum albumin (BSA), and poly(GluNa, Tyr) (PGT) were purchased from Sigma Chemical Corp. Recombinant human IGF-I was purchased from Pepro Tech, Inc. Trypsin (225 U/mg) was purchased from Worthington Biochemical Corp. and fetal calf serum (FCS) was purchased from Dutscher. *AmpliTaq* DNA polymerase was purchased from Perkin-Elmer.

Cell culture

BC1 and B9 cells have been cloned from a human hepatocarcinoma (Le Jossic *et al.*, 1995). Cells were grown and maintained at 37°C in a humidified 5% CO_2/95% air atmosphere in complete medium (3/4 MEM, 1/4 Medium 199, 10% FCS, 2 mM L-glutamine, $NaHCO_3$ (2.2 g/l), BSA (1 g/l), 10^{-7} M hydrocortisone, penicillin/streptomycin) without or with 1 μM insulin. Medium was replaced every two days. After trypsinization (0.025% trypsin, 0.5 mM EDTA), BC1 cells were plated at a density of approximately 2×10^6 cells per 25 cm^2 flasks. HepG2 cells were obtained from American Type Culture Collection and cultured in complete medium (DMEM, 10% FCS, 2 mM L-glutamine, $NaHCO_3$ (2.2 g/l), penicillin/streptomycin) as described above.

RNA extraction and Northern blot analysis

BC1, B9 and HepG2 cells were washed twice with ice-cold phosphate buffer saline (PBS) and total cellular RNA was extracted by RNAzol^{TM}B according to the procedure of Chomczynski & Sacchi (1987). Poly(A)$^+$-containing mRNA was prepared by chromatography on oligo(dT)-cellulose using the Poly(A) Quik mRNA kit (Stratagene). The RNA (15 μg) was denatured in 5% formaldehyde and electrophoresed on 1% agarose gels (Thomas, 1980) and transferred to Hybond-N$^+$ nylon membranes (Amersham). After alkali fixation (0.05 N NaOH), membrane filters were prehybridized for 5–6 h at 65°C in a solution containing 10% dextran sulfate, 5 × Denhardt's reagent (1 × = 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 5 × SSPE (1 × = 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA (pH 7.4)), 0.5 mg/ml heparin, and 50 μg/ml denatured salmon sperm. A 1.1 kb *PstI* fragment (pBR 322) of the human albumin gene, a 1.0 kb *PstI* fragment (pBR 322) of the human transferrin gene, and a full length 4.5 kb *EcoRI* and *XbaI* fragment (pECE) of the human insulin receptor cDNA were labelled with [α-^{32}P]dCTP using a Megaprime labelling-system kit. Denatured ^{32}P-labelled cDNA probes (1.5×10^6 c.p.m./ml) were added and hybridization was carried out for 16–20 h at 65°C. The membranes were washed once for 15 min in 5 × SSPE, 0.1% SDS at 65°C, once for 15 min in 1 × SSPE, 0.1% SDS at 65°C, and once for 1 h in 0.5 × SSPE, 0.1% SDS at 65°C, and autoradiographed with Hyperfilm-MP (Amersham) using an intensifying screen at −80°C. RNA was quantified by densitometric scanning. Relative values were calculated by normalizing to the amount of 28S rRNA on the same filters, achieved by hybridization with a corresponding oligonucleotide probe (Barbu & Dautry, 1989) labelled with T4 kinase and [γ-^{32}P]ATP.

Western blot analysis

BC1 cells in 25 cm^2 flasks were incubated in 4 ml of serum-free medium for 24 h. A 45-μl aliquot of the culture medium was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% (w/v) acrylamide and 0.1% (w/v) SDS) according to Laemmli (1970). Proteins were electrotransferred to nitrocellulose membranes (Hybond^{TM}-ECL Western Amersham). Blots were then blocked for 16 h at 4°C with 5% dried milk and 0.05% Nonidet in Tris buffered saline (TBS) (20 mM Tris-base-137 mM NaCl, pH 7.6) containing 0.1% Tween-20. Blots were then incubated for 2 h at room temperature in the same buffer containing a 1/1000 dilution of either a polyclonal human and anti-albumin peroxidase-conjugated antibody or a polyclonal human anti-transferrin antibody. For the latter, after 3 × 15-min washes with TBS containing 0.1% Tween-20, filters were incubated for 1 h at room temperature with a 1/6000 dilution of a goat anti IgG-peroxidase-conjugated antibody. Detection of immunoreactive proteins was performed with the ECL Western blotting kit (Amersham).

Binding assays

Binding experiments were performed as previously described (Baron-Delage *et al.*, 1994). FCS-deprived BC1 cells in 12-well plates were incubated at 4 h at 15°C in 0.5 ml of binding assay buffer (100 mM HEPES pH 7.5, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 15 mM Na-Acetate, 10 mM glucose, 1% BSA), containing 10^{-12} M [^{125}I]Insulin or 3.4×10^{-11} M [^{125}I]IGF-I, increasing concentrations of unlabeled insulin (0-2×10^{-7} M) or IGF-I (0-2×10^{-6} M). After three washing with 2 ml of ice-cold PBS, cells were dissolved in 250 μl of 20% KOH for 1 h and counted for radioactivity. Nonspecific binding of insulin or IGF-I was determined in the presence of 1 μM unlabeled ligand and was subtracted from each data point. Protein was determined by the method of Lowry *et al.* (1951) using BSA as a standard. Data were analysed with a computational program (RIACALC Lab Manager (LKB-Pharmacia)).

Reverse transcription and amplification of IR cDNA

First-strand cDNA was prepared by reverse transcription using 0.6 μg of poly(A)$^+$ mRNA in a volume of 20 μl (100 ng of the 3′ oligonucleotide, 40 units of RNAsin, 200 units of Mu-MLV reverse transcriptase, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTPs) at 37°C for 1 h. DNA/RNA hybrids were denatured at 95°C for 2 min.

Oligonucleotide primers spanning nucleotides of exon 9 (114-134) (sense primer, 5′-GGGAGAGGCAGGCGGAA-GACA-3′) and exon 12 (173-193) (antisense primer, 5′-GCGATAGCCCGTGAAGTGTCG-3′) were used to amplify a region of the human IR cDNA that overlapped the alternatively spliced exon 11 (Seino *et al.*, 1990). These primers generate either a 446- or a 482-bp fragment following amplification of the mRNA encoding the A isoform (exon 11−) or B isoform (exon 11+), respectively. The Glut 1 primer pair consisted of oligonucleotides spanning nucleotides 296-315 (sense primer, 5′-ATGAACCTGCTG-GCCTTCGT-3′) and 540-559 (antisense primer, 5′-CCAC-AGGTCCTTGTTGCCCA-3′) of the ubiquitous glucose transporter which generate a fragment of 264 bp following amplification (numbering as in Mueckler *et al.*, 1985). 5 μl of the cDNA synthesis reaction were used for PCR amplification in a 25 μl final volume (100 ng of each oligonucleotide primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTPs, 2 units of Taq DNA polymerase, and 1 μCi of [α-^{32}P]dCTP). Thirty cycles of amplification were performed using a DNA thermal cycler (Perkin-Elmer-Cetus Instruments). Each cycle consisted of a 30 s denaturation at 94°C, a 1 min annealing at 58°C, and a 1 min 30 s extension at 72°C. The number of cycles was optimized to ensure that the amplification lay within the exponential phase (data not shown). The products of PCR amplification were resolved by electrophoresis on 2% agarose gels, transferred to Hybond-N$^+$ nylon membranes and autoradiographed.

Autophosphorylation and tyrosine kinase activity of purified insulin receptor

Insulin receptor partial purification was achieved by lectin affinity chromatography as previously described (Caron *et al.*, 1994). BC1 cells in 2 × 225-cm^2 flasks were deprived of FCS and insulin for 48 h, washed with ice-cold PBS, and lysed in 4 ml of ice-cold solubilization buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 0.2 mM PMSF, 0.15 M NaCl, 5% glycerol and 1200 U/ml aprotinin). After gentle mixing for 1 h, the cell lysate was centrifuged at 100 000 *g* for 1 h. The supernatant was applied to a wheat germ agglutinin agarose column and recycled three times. The column was washed extensively with 0.1% Triton X-100 in 50 mM HEPES pH 7.4. Bound glycoproteins were eluted with 0.3 M *N-Acetyl*-D-glucosamine in washing buffer. Protein was measured by the Bradford protein assay (Bio-Rad) using BSA as standard. For analysis of autophosphorylation of the β subunit of the insulin receptor (IR), aliquots of WGA-purified extracts were treated without or with 1 μM insulin in 100 μl (final volume) of 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100. After 40 min at 22°C, 2 mM $MnCl_2$, 25 μM [γ-^{32}P]ATP were added for 10 min. The reaction was stopped by 20 μl of a blocking solution containing 10 mM EDTA, 100 mM NaF, 20 mM sodium pyrophosphate and 250 μg/ml BSA and proteins was precipitated with 5% trichloroacetic acid at 4°C for 15 min. After solubilization of the pellet with electrophoresis sample buffer, labelled IRs were analysed by SDS-PAGE (7.5%) and autoradiography. IR tyrosine kinase activity was evaluated by measuring the phosphorylation of the exogenous substrate PGT. The phosphorylation reaction was carried out as described above except that 0.7 mg/ml PGT, 35 mM $MgCl_2$ and 50 μM ATP were added in a final volume of 150 μl. 70 μl of the reaction mixture were spotted onto Whatman P81 paper squares which were washed 5 times for 5 min in 75 mM phosphoric acid, once with 95% ethanol and dried. The incorporated radioactivity was determined by liquid scintillation counting.

Insulin stimulation of glucose incorporation into glycogen

Glycogen synthesis was evaluated by measuring the incorporation of D-[U-^{14}C]glucose into cellular glycogen as previously described (Capeau *et al.*, 1984). Briefly, BC1 cells in 12-well plates were deprived of FCS and insulin for 48 h and then incubated in 0.5 ml medium containing 0.6 μCi/ml of [U-^{14}C]glucose in the presence of increasing concentrations (0–10^{-6} M) of insulin at 37°C for 1 h. Cells were washed twice with ice-cold PBS and glycogen was extracted in 20% KOH. After boiling the sample, glycogen was ethanol-precipitated, recovered in a centrifugation pellet and counted for radioactivity in aqueous scintillation fluid.

Acknowledgements

We thank Dr Christiane Brahimi-Horn for reviewing the manuscript. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, INSERM, U. 402 (France) and the Association pour la Recherche contre le Cancer (A.R.C., Villejuif).

References

Accili, D. & Taylor, S.I. (1991). *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4708-4712.

Barba, V. & Dautry, F. (1989). *Nucleic Acids Res.*, **17**, 7115.

Baron-Delage, S., Capeau, J., Barbu, V., Chastre, E., Levy, P., Gespach, C. & Cherqui, G. (1994). *J. Biol. Chem.*, **269**, 18686-18693.

Benecke, H., Flier, J.S. & Moller, D.E. (1992). *J. Clin. Invest.*, **89**, 2066-2070.

Bernuau, D., Poliard, A. & Feldmann, G. (1988). *Hepatology*, **8**, 97-1005.

Bissell, D.M., Arenson, D.M., Maher, J.J. & Roll, F.J. (1987). *J. Clin. Invest.*, **79**, 801-812.

Capeau, J., Flaig-Staedel, C., Beck, J.P. & Picard, J. (1984). *Endocrinology*, **115**, 654-661.

Caron, M., Reynet, C., Wicek, D., Picard, J., Cherqui, G. & Capeau, J. (1994). *Metabolism*, **43**, 757-765.

Caron, J.M. (1990). *Mol. Cell. Biol.*, **10**, 1239-1243.

Cereghini, S., Yaniv, M. & Cortese, R. (1990). *EMBO J.*, **9**, 2257-2263.

Chan, K., Kost, D.P. & Michalopoulos, G. (1989). *J. Cell. Physiol.*, **141**, 584-590.

Chomczynski, P. & Sacchi, N. (1987). *Anal. Biochem.*, **162**, 156 159.

Crettaz, M. & Kahn, C.R. (1983). *Endocrinology*, **113**, 1201-1209.

De Juan, C., Benito, M. & Fabregat, I. (1992). *J. Cell. Physiol.*, **152**, 95-101.

Gaben-Cogneville, A.M., Poussin, B., Chamblier, M.C., Forgue-Lafitte, M.E. & Rosselin, G. (1988). *Biochim. Biophys. Acta.*, **968**, 231-238.

Guguen, C., Gregori, C. & Shapira, F. (1975). *Biochimie*, **9**, 1065 1071.

Guguen-Guillouzo, C., Bourel, M. & Guillouzo, A. (eds.) (1986). In: *Progress in liver diseases*. Grune & Stratton. Academic press. pp. 33 50.

Hauner, H. (1990). *Endocrinology*, **127**, 865 872.

Hoffmann, B., Piasecki, A. & Paul, D. (1989). *J. Cell. Physiol.*, **139**, 654-662.

Jefferson, L.S., Liao, W.S.L., Peavy, D.E., Miller, T.B., Appel, M.C. & Taylor, J.M. (1983). *J. Biol. Chem.*, **258**, 1369-1375.

Koontz, J.W. & Iwahashi, M. (1981). *Science*, **211**, 947-949.

Kosaki, A.K. & Webster, N.J.G. (1993). *J. Biol. Chem.*, **268**, 21990-21996.

Laemmli, U.K. (1970). *Nature*, **227**, 680-685.

Le Jossic, C., Glaise, D., Diot, C., Dezier, J.F., Fautrel, A., Corcos, L. & Guguen-Guillouzo, C. (1995). *Euro. J. Biochem.*, in press.

Lloyd, C.E., Kalinyak, J.E., Hutson, S.M. & Jefferson, L.S. (1987). *Am. J. Physiol.*, **252**, C205-C214.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). *J. Biol. Chem.*, **193,** 265–275.
Marsden, E.R., Hu, Z., Fujio, K., Nakatsukasa, H., Thorgeirsson, S.S. & Evarts, R.P. (1992). *Lab. Invest.*, **67,** 427–433.
McClain, D.A. (1991). *Mol. Endocrinol.*, **5,** 734–739.
Messina, J.L. (1992). *Biochim. Biophys. Acta.*, **1137,** 225 230.
Mosthaf, L., Grako, K., Dull, T.J., Coussens, L., Ullrich, A. & McClain, D.A. (1990). *EMBO J.*, **9,** 2409–2413.
Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E. & Lodish, H.F. (1985). *Science*, **229,** 941–945.
Mueller, C.R. (1992). *DNA and Cell Biol.*, **11,** 559–566.
Nagy, P., Bisgaard, H.C. & Thorgeirsson, S.S. (1994). *J. Cell. Biol.*, **126,** 223–233.
Ng, K.H., Maigné, J., Fougère-Deschatrette, C., Poupon, M/F. & Deschatrette, J. (1993). *Exp. Cell. Res.*, **207,** 48 56.
Reif, S., Sykes, D., Rossi, T. & Weiser, M.M. (1991). *Hepatology*, **15,** 310 315.
Rice, K.M., Lienhard, G.E. & Garner, C.W. (1992). *J. Biol. Chem.*, **267,** 10163–10167.
Rubin, C.S., Hirsch, G.E., Fung, C. & Rosen, O.M. (1978). *J. Biol. Chem.*, **253,** 7570–7578.
Saad, M.J.A., Folli, F., Araki, E., Hashimoto, N., Csermely, P. & Kahn, C.R. (1994). *Mol. Endocrinol.*, **8,** 545–557.
Sadowski, H.B., Wheeler, T.T. & Young, D.A. (1992). *J. Biol. Chem.*, **266,** 4722–4731.
Seino, S. & Bell, G.I. (1989). *Biochem. Biophys. Res. Commun.*, **159,** 312–316.
Seino, S., Seino, M. & Bell, G.I. (1990). *Diabetes*, **39,** 123–128.
Sell, S.M., Reese, D. & Ossowski, V.M. (1994). *J. Biol. Chem.*, **269,** 30769–36772.
Shimizu, Y. & Shimizu, N. (1986). *J. Biol. Chem.*, **261,** 7342–7346.
Smith, P.J., Wise, L.S., Berkowits, R., Wan, C. & Rubin, C.S. (1988). *J. Biol. Chem.*, **263,** 9402 9408.
Straus, D.S. & Takemoto, C.D. (1987). *J. Biol. Chem.*, **262,** 1955–1960.
Thomas, P.S. (1980). *Proc. Natl. Acad. Sci. U.S.A.*, **77,** 5201 5205.
Thompson, D., Harrison, S.P., Evans, S.W. & Whicher, J.T. (1991). *Cytokine*, **3,** 619–626.
Yamaguchi, Y., Flier, J.S., Benecke, H., Ransil, B.J. & Moller, D.E. (1993). *Endocrinology*, **132,** 1132–1138.
Yamaguchi, Y., Flier, J.S., Yokota, A., Benecke, H., Backer, J.M. & Moller, D.E. (1991). *Endocrinology*, **129,** 2058 2066.