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Expression and regulation of the insulin-like growth factor-1 receptor by growing and quiescent H4IIE hepatoma

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Abstract

Recent evidence that insulin-like growth factor-1 (IGF-1) influences certain properties of H4IIE hepatoma cells independent of insulin led us to examine whether H4IIE cells express IGF-1 receptors. Competitive binding experiments demonstrated IGF-1, but not insulin or IGF-II, could compete with [^{125}I]IGF-1. Chemical crosslinking detected a protein with an apparent mass of 175 kDa and its identity as the IGF-1 receptor α-subunit was confirmed by Western blotting. The apparent molecular mass of this protein decreased to 135 kDa following deglycosylation. Immunofluorescence microscopy verified that both insulin and IGF-1 receptors were present, although measurement of IGF-1 receptor quantity revealed they were less abundant than insulin receptors. Binding of IGF-1 was low in growing cells and higher in a quiescent cell population. Scatchard analysis confirmed that receptor density was increased in non-growing H4IIE cells while there was no apparent difference in receptor affinity. Western blot analysis and RT-PCR revealed that both protein and mRNA levels were elevated as cell growth ceased. Interestingly, addition of insulin to quiescent H4IIE cells, which stimulates cell proliferation, further increased IGF-1 receptor protein levels with a peak at 12–24 h. Distinct modes of regulating IGF-1 receptor expression are indicated.

Keywords: Insulin-like growth factor-1; Hepatoma

1. Introduction

Insulin-like growth factor-1 (IGF-1) exerts its diverse biological effects by binding to specific receptors located on the cell surface. The IGF-1 receptor is a heterotetramer arranged in an $\alpha_2\beta_2$ configuration held together by disulfide bridges [1]. The ligand binding domain is formed by the extracellular α-subunits and signals are conveyed into the cell via the transmembrane domain of the β-subunit. Activation of the tyrosine kinase domain located on the intracellular portion of the β-subunit initiates the intracellular signalling cascade that mediates the cellular response to IGF-1. Due to a high degree of structural similarity with the insulin receptor which results in heterologous binding of insulin to the IGF-1 receptor (and vice versa) [2], it has been difficult to identify the biological effects mediated solely by the IGF-1 receptor. Thus, while insulin has been designated a metabolic hormone and IGF-1 is considered a mitogenic factor, crossreactivity with respect to receptor occupancy has obscured these distinctions.

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Although the liver is the primary source for circulating IGF-1, there is little evidence that IGF-1 influences either hepatic metabolism or growth. For instance, hepatic regeneration following injury, an event that would most likely be associated with a mitogen such as IGF-1, is directed by insulin [3]. A lack of IGF-1 receptors on hepatocytes would explain these observations, and an absence of these receptors has been reported in several studies [2,4]. On the other hand, there are published reports indicating that IGF-1 receptors, although in lesser numbers, are expressed by hepatocytes [5–7]. Not surprisingly, a clear consensus has not yet been reached on this issue.

This laboratory has used the H4IIE hepatoma cell line [8] to examine the intracellular systems mediating insulin-dependent cell growth [9,10]. Over the course of our investigations, we have noted that IGF-1, although ineffective as a mitogen, is capable of stimulating several growth-associated processes [11]. Reports that the parental Reuber H-35 hepatoma cell line lacks IGF-1 binding activity [12,13], however, suggest that H4IIE cells might also be deficient of IGF-1 receptors. A comprehensive examination of H4IIE cells was conducted to determine whether IGF-1 receptors are present. Our investigation revealed that IGF-1 receptors are expressed by H4IIE cells, and that this expression is modulated during changes in growth state and by insulin.

2. Materials and methods

2.1. Cell culture

Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were maintained in α-modified Eagle's medium (α-MEM, Gibco-BRL) containing 10% fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 μg/ml penicillin. Quiescence was achieved by placing the cells into serum-free medium for 72 h [10].

2.2. Ligand binding

H4IIE cells were plated on 12-well culture dishes and placed into serum-free media when 60% confluency was reached. To measure ligand binding [14], the media was removed, the cells were washed twice with ice-cold PBS (150 mM NaCl, 10 mM KPO_4 pH 7.5) and the original media was replaced with 0.25 ml α-MEM containing 20 mM HEPES pH 7.4 and 0.01% bovine serum albumin. The competitor peptide (insulin, IGF-II or IGF-1) was added prior to addition of the radiolabeled ligand [^{125}I]IGF-1 (80 000 cpm). The cells were incubated with continuous slow agitation for 2 h at 4°C. The cells were subsequently washed three times with ice-cold PBS and solubilized by incubating with 0.5 ml NaOH (0.3 M) for 30 min at ambient temperature. The samples were transferred to vials and mixed with scintillation cocktail (Aquasol, New England Nuclear) for radioisotopic detection. Non-specific binding was measured in the presence of excess (10 μM) unlabeled IGF-1.

2.3. Affinity labeling

H4IIE cells were incubated with [^{125}I]IGF-1 (New England Nuclear, 25 μCi/ml) as described for ligand binding. The cells were rinsed twice with 1 ml ice-cold PBS and placed into 0.25 ml α-MEM containing 20 mM HEPES pH 7.4 and lacking bovine serum albumin. Disuccinimidyl suberate (DSS), freshly prepared in DMSO, was added to a final concentration of 0.5 mM [15]. The samples were maintained on ice for 30 min. The cells were subsequently washed three times with 1 ml PBS and scraped from the plates using Cell Lifters (Costar). The cells were collected by centrifugation (5 min, 3000×*g*) and suspended in 25 μl 2×SDS/gel loading buffer (1×buffer = 62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 0.005% Bromophenol blue, 5% β-mercaptoethanol). The samples were briefly sonicated and 15 μl loaded onto a 7.5% polyacrylamide gel. The gel was dried and exposed to Reflection autoradiography film (New England Nuclear) with one intensifying screen.

2.4. Western blotting

Cell extracts were prepared in 12-well dishes by addition of 150 μl 2×SDS/gel loading buffer. The samples were briefly sonicated, centrifuged for 15 min at 12 000×*g* and 25 μl loaded onto 7.5% polyacrylamide gels. Electrophoresis was conducted at constant current and the proteins transferred to

PVDF membrane in 20% methanol, 25 mM Tris, 130 mM glycine. Membranes were treated for 60 min at room temperature with blocking solution (3% BSA in TBS-T (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% Tween 20)). Primary antibody was added in fresh blocking solution and incubated for 60 min at 37°C. Membranes were subsequently washed three times over 15 min with TBS-T and incubated for an additional 60 min at 37°C in blocking solution with secondary antibody (1:10 000 diluted anti-rabbit-HRP). HRP was detected using the ECL chemiluminescent system (Amersham) after washing the membranes five times over 30 min with TBS-T. All antibodies were purchased from Santa Cruz Biotechnology.

2.5. Immunocytochemistry

Quiescent cells, prepared on glass slides (Superfrost Plus, Fisher Scientific), were washed twice with cold PBS and fixed for 10 min at 4°C in 1% paraformaldehyde solution [16]. The cells were subsequently permeabilized by treatment for 8 min at 4°C with 0.1% Triton X-100 (in PBS), rinsed thoroughly with PBS, and incubated for 30 min at ambient temperature with TBS-T containing 3% BSA. Incubations of 30 min with primary and secondary antibodies (diluted with 1% BSA in TBS-T) were used to visualize the cells for fluorescence microscopy as described in the figure legend.

2.6. Protein deglycosylation

H4IIE cells were scraped from 150-mm culture dishes in 3 ml PBS using Cell Lifters. Cells were collected by centrifugation (5 min $2000\times g$), and the pellet suspended with 10 vols. PBS. A 100-μl aliquot of the cell suspension was heated for 2 min at 100°C and allowed to cool. From this sample, 10 μl was removed and combined with 5 μl 5% NP-40 and 15 μl *N*-glycanase (200 U/ml peptide-*N*-glycosidase F, Oxford Glycosciences), and this mixture was then incubated at 37°C for 18 h. The sample was mixed with an equal volume of 2×SDS/loading buffer and analyzed by Western blot after electrophoresis in a 7.5% polyacrylamide gel.

2.7. Receptor quantification

H4IIE cells were harvested from 150-mm culture dishes as described in the previous section and extracted with 2.5 pellet volumes of PBS containing 1% Triton X-100. Nuclei were removed by centrifugation ($10\,000\times g$, 15 min). Serial dilutions of the supernatant were mixed with 2×SDS/gel loading buffer and applied to a 7.5% polyacrylamide gel. Serial dilutions of the control peptide (used as antigen to raise the antibody) were applied to the same gel after the samples had migrated 50% of the distance through the gel. When completed, the samples were transferred to PVDF membrane and relative amounts of IGF-1 and insulin receptor β-subunits in the cellular material were determined by Western blotting using the intensity of the control peptide for comparison. Protein concentration was determined with the BCA protein assay system (Pierce).

2.8. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from adherent cells using Trizol (Gibco-BRL) as described previously [17]. The RNA precipitate was collected by centrifugation, washed (70% ethanol) and dissolved in 25 μl RNase-free water. RNA concentration was estimated by absorbance at OD_{260}. Reverse transcription of 1 μg of RNA was conducted according to the protocol recommended for the GeneAmp kit (Perkin Elmer-Cetus). For PCR, 25 ng sense and antisense primers and 1.25 U Amplitaq *Taq* DNA polymerase were added to each RT-reaction (50 μl final volume). Amplification was conducted over 35 cycles using a three-step program (1 min at 95°C, 1 min at 55°C, 1 min at 72°C) that was concluded with 7 min at 72°C. Samples were analyzed by electrophoresis on 1.7% agarose gel and visualized with SYBR Green I (Molecular Probes). Primers for the IGF-1 receptor [18], GAPDH [16] and ribosomal protein L32 (forward primer, CAGGGTGCGGAGAAGGTT; reverse primer, GCGTTGGGATTGGTGACT; based on the sequence reported by Dudov and Perry [19]) were prepared using an Oligo1000 DNA Synthesizer (Beckman).

3. Results

Although liver contains receptors for IGF-1 as demonstrated by both ligand binding and affinity crosslinking experiments [5,6], the Reuber H-35 hepatoma from which the H4IIE cell line was derived apparently lacks IGF-1 receptors [12,13]. Since we have observed that certain intracellular events are stimulated by IGF-1 [11], and there have been no reports either verifying or refuting the presence of IGF-1 receptors on H4IIE cells, we investigated this question directly using [^{125}I]IGF-1 as a ligand.

Binding of IGF-1 to H4IIE cells was observed under conditions previously defined for fibroblasts and smooth muscle cells [14] which used low temperature to minimize receptor internalization. Radiolabeled IGF-1 was displaced by addition of unlabeled IGF-1 indicating competition for binding sites (Fig. 1). Insulin, which has a lower binding affinity for the IGF-1 receptor, was unable to compete with the IGF-1. IGF-II, on the other hand, competed with the ligand, but only at higher concentration. These results indicate that H4IIE cells have high affinity binding sites for IGF-1 on their plasma membranes.

Chemical crosslinking of [^{125}I]IGF-1 to H4IIE cells with DSS demonstrated this ligand was bound to a protein of 175 kDa as determined by SDS-PAGE under reducing conditions (Fig. 2). This more closely approximates the 135 kDa molecular mass typically reported for the IGF-1 receptor α-subunit than the IGF-II receptor which migrates with an apparent molecular mass of 250 kDa [2,20]. The binding specificity was verified by the ability of competitor IGF-1 to reduce the labeling intensity, while insulin and IGF-II were 10–100-fold less effective in displacing the label.

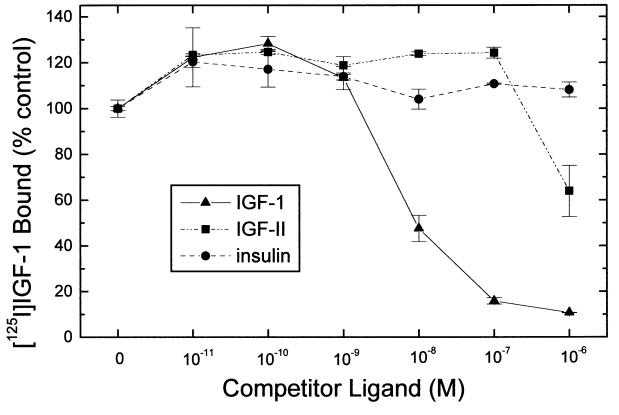

Fig. 1. Competitive ligand binding of IGF-1 to H4IIE hepatoma cells. Quiescent H4IIE cells were incubated with [^{125}I]IGF-1 for 2 h in the presence or absence of either unlabeled insulin, IGF-II or IGF-1. The bound [^{125}I]IGF-1 was quantified by scintillation counting after extensive washing to remove unbound label. Each data point represents the mean ± S.E. of experiments conducted in triplicate.

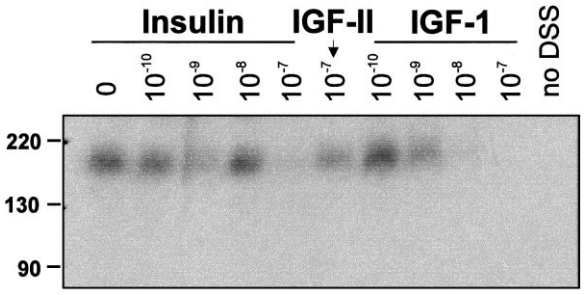

Fig. 2. Receptor crosslinking to IGF-1. [^{125}I]IGF-1 was incubated with the cells for 2 h in the presence or absence of unlabeled insulin, IGF-II or IGF-1 (at the indicated concentrations) and unbound label removed by washing with PBS. Crosslinking agent, 0.5 mM DSS, was added in fresh binding buffer excluding bovine serum albumin. After 30 min on ice, the cells were harvested and the proteins crosslinked to IGF-1 identified by autoradiography after SDS-PAGE. The molecular mass of the labeled protein was calculated using prestained markers (their position is indicated on the left side) run simultaneously in an adjacent lane. Duplicate experiments provided identical results.

To establish that the labeled protein was indeed the α-subunit of the IGF-1 receptor, H4IIE cell extracts were examined by Western blotting. It was observed, using specific antibodies that did not cross-react with the corresponding insulin receptor subunits, that both the α-subunit and β-subunit of the IGF-1 receptor were present (Fig. 3A,B). Furthermore, the apparent molecular mass of the α-subunit was calculated as 170 kDa, closely matching the value observed in crosslinking experiments (Fig. 2). The β-subunit migrated with a molecular mass of 95 kDa, identical to published values [1]. Although the apparent molecular mass of 175 kDa for the α-subunit (Fig. 3A) is higher than the 135 kDa usually quoted, variations in molecular mass have been detected previously and these differences can be accounted for by the degree of N-linked glycosylation [21]. To verify that the IGF-1 receptor of H4IIE cells is similarly modified, cells were incubated with glycosylase F to remove N-linked polysaccharides. Western blot anal-

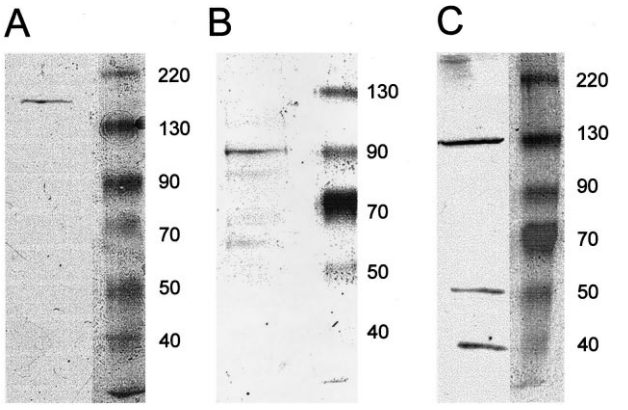

Fig. 3. Western blot analysis of IGF-1 receptors. (A) Lysates of quiescent H4IIE cells were applied to 7.5% polyacrylamide gels and after electrophoresis transferred to PVDF membrane. The blot was probed with antibody specific for the IGF-1 receptor α-subunit (N-20 region). (B) The same sample was applied to a 10% polyacrylamide gel and, following transfer to PVDF membrane, probed with antibody specific for the β-subunit (C-20 region) as indicated. (C) Deglycosylation was conducted as described in Section 2, and migration of the IGF-1 receptor α-subunit was monitored by Western blot after electrophoresis in 10% polyacrylamide. The applicable prestained molecular mass markers (Benchmark Protein Ladders, Gibco-BRL) are shown in each panel.

ysis of the samples revealed that the molecular mass of the α-subunit was reduced to approximately 135 kDa by this treatment (Fig. 3C).

Our results suggest H4IIE hepatomas express receptors for both IGF-1 and insulin. Immunofluorescence microscopy verified that both IGF-1 and insulin receptors can be detected using a combination of both primary and secondary antibodies, but not with either alone (Fig. 4A–D). Specificity of the individual antibodies for either the IGF-1 or the insulin receptor β-subunit was established by preincubating the antibodies with blocking peptide, which resulted in a significant reduction in intensity (Fig. 4E,F). While this approach provided a direct visualization of the receptors, it could not be used to infer relative amounts. To compare insulin and IGF-1 receptor numbers, the control (i.e. antigenic or blocking) peptide was used as the vehicle to translate band intensity on a Western blot to actual concentration. The protocol that was employed (see Section 2) established that 3.2 ng insulin receptor/mg protein was present in a cell lysate which contained 0.55 ng IGF-1 receptor/mg protein (Table 1). The relative

Table 1
Quantification of insulin and IGF-1 receptor densities

	Receptor	
	Insulin (ng/mg total protein)	IGF-1 (ng/mg total protein)
Concentration	3.21 ± 0.27	0.55 ± 0.12

Quiescent H4IIE cells were harvested and extracted for SDS-polyacrylamide gel electrophoresis. Control peptide was applied to the same gel in adjacent wells once electrophoresis of the initial cell samples was half completed. The insulin and IGF-1 receptor analysis were conducted on separate gels. After transfer to PVDF membrane, antibodies to the receptor β-subunit were used to probe the membrane and the band intensities quantified by scanning densitometry. The standard curve produced using the control peptide (100 μg/ml stock concentration) was used to calculate the receptor concentration in each sample. The protein concentration of the cell lysate was determined using the BCA protein assay.

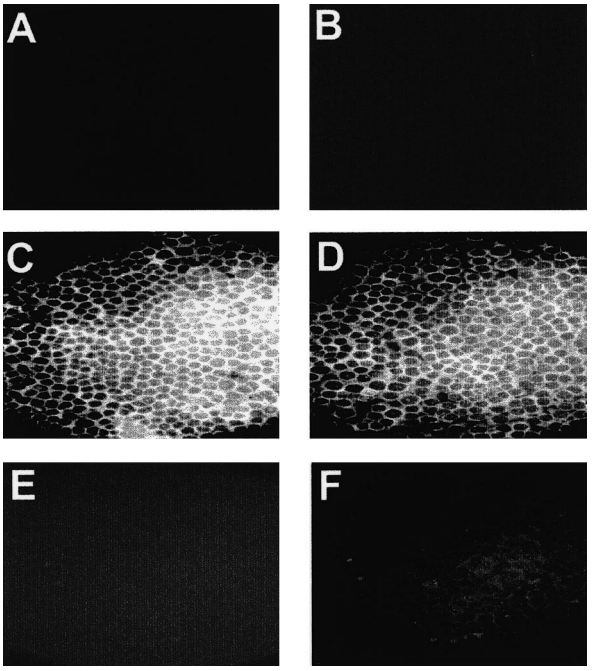

Fig. 4. Immunocytochemical analysis of H4IIE hepatoma IGF-1 and insulin receptors. Quiescent cells were fixed and stained with antibodies (diluted 1:200 in TBS-T plus 1% BSA) specific for either insulin or IGF-1 receptor β-subunits followed by anti-rabbit IgG conjugated to Cy3. (A) and (B) show the results of staining in the absence of primary (A) or secondary (B) antibodies, respectively. Staining for the insulin (C,E) and IGF-1 (D,F) receptors were conducted without (C,D) or with (E,F) a 20 min incubation in the presence of 250 μg control peptide. Each panel includes a single colony of H4IIE cells photographed with a 20× objective and 1.67× phototube.

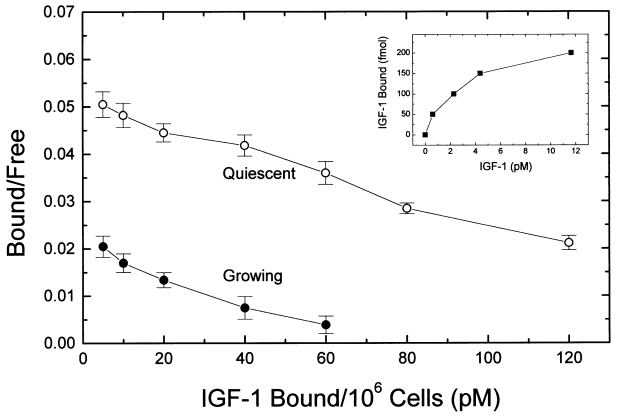

Fig. 5. IGF-1 binding characteristics of growing and quiescent H4IIE cells. A Scatchard analysis of IGF-1 binding was made using both proliferating and quiescent (72 h in serum-deficient media) H4IIE cells. All values, means ± S.E. of triplicate binding experiments, were normalized to cell number which was determined in two independent wells on each culture dish. Duplicate experiments yielded the same results for K_d and B_{max}. The inset shows the saturation curve for these data.

IGF-1 receptor density is thus 17% of the insulin receptor density.

Considering the close relationship with H4IIE cells, the lack of observable IGF-1 binding in Reuber H-35 hepatomas [12,13] contrasts markedly with the observations reported herein. We have previously noted with the angiotensin type 2 receptor, however, that receptor binding can vary in accordance with growth state [22]. It is therefore plausible that our experiments and those of Krett et al. [13] were conducted under sufficiently different conditions that divergent results were obtained. To address this issue, the IGF-1 binding characteristics of growing and quiescent H4IIE cells were compared. Ligand binding revealed that both growing and quiescent H4IIE cells exhibited similar binding characteristics for IGF-1 (Fig. 5), with cells under both conditions yielding a binding constant (K_d) of between 3.5 and 4 nM (growing, 3.55 ± 0.39; quiescent, 3.93 ± 0.24). In contrast, the receptor density of quiescent cells (59 600 sites/cell) was significantly higher than that observed for growing cells (21 400 sites/cell), based on B_{max} values of 197.9 ± 4.6 and 71.0 ± 3.8 pM/10^6 cells, respectively. While evidence for functional IGF-1 receptors was obtained with both growing and quiescent cells, an increase in IGF-1 receptor expression by quiescent H4IIE cells was indicated.

To define the changes in IGF-1 binding more directly, as well as to establish that IGF-1 binding resulted from an association with the IGF-1 receptor, levels of IGF-1 receptor protein and mRNA were measured. Western blot analysis using an antibody specific for the IGF-1 receptor β-subunit revealed that receptor levels increased as cells entered a quiescent state (Fig. 6). A parallel rise in IGF-1 receptor mRNA was detected by reverse transcriptase-PCR amplification (Fig. 7). These results strongly support the ligand binding data which indicate IGF-1 receptor levels are higher in quiescent H4IIE cells.

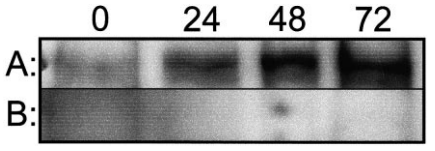

Fig. 6. Western blot analysis of the IGF-1 receptor β-subunit. Growing H4IIE cells or cells that had been placed into serum-deficient media for 1, 2 or 3 days were harvested by addition of SDS-gel loading buffer. Samples with equivalent protein concentration (15 μg) were analyzed by Western blotting using an antibody directed to the C-terminus of the IGF-1 receptor as described in Section 2. (A) Results using the antibody directly. (B) Results obtained when the antibody was preincubated for 30 min with blocking peptide. Two additional experiments produced the same result.

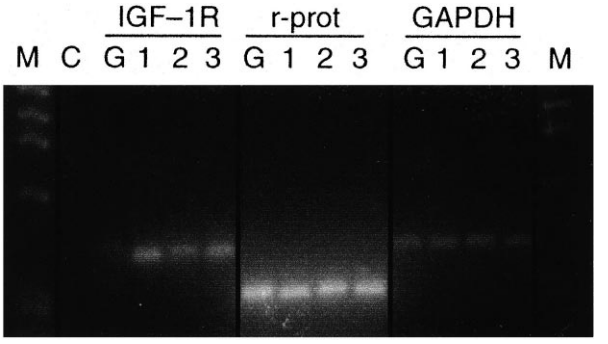

Fig. 7. RT-PCR analysis of IGF-1 receptor mRNA levels in H4IIE hepatoma cells. Total RNA was isolated from proliferating (G) H4IIE cells or cells that had been placed into serum-free media for 24 (1), 48 (2) or 72 (3) h. The RNA was reverse transcribed (lane C had no RNA added) and subsequently PCR amplified using primers specific for the IGF-1 receptor (IGF-1R), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ribosomal protein L32 (r-prot) as described in Section 2. The products were resolved by electrophoresis on 1.7% agarose gels and visualized using SYBR Green I DNA stain. DNA size markers (*Hae*III digested ϕX174 DNA) are shown in lane M and cover the range from 1353 to 194 nucleotides. Identical results were obtained in two independent experiments.

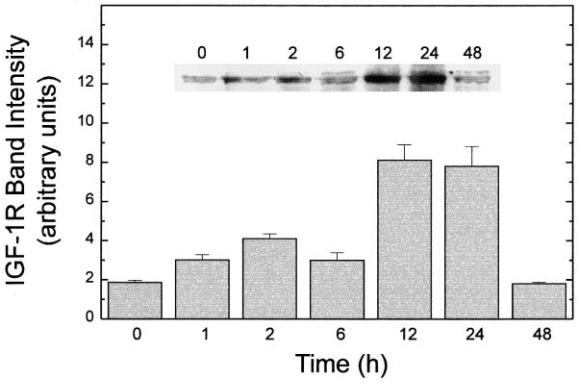

Fig. 8. Modulation of IGF-1 receptor levels by insulin. Quiescent H4IIE cells (72 h in serum-deficient media) were stimulated with 1 μM insulin and harvested at the indicated times by addition of SDS-gel loading buffer. Aliquots of 15 μg protein were analyzed by Western blot using an antibody to the IGF-1 receptor α-subunit. Band intensities from three independent experiments (one example is provided as an inset) were quantified by scanning densitometry and plotted as means ± S.E.

Insulin modulates insulin and IGF-1 receptor expression in numerous systems where it does not have a mitogenic effect. In H4IIE hepatomas, however, where insulin functions as a mitogen as well as a metabolic hormone [10,23], it is difficult to distinguish whether the changes in IGF-1 receptor levels between proliferating and quiescent cells reflect a change in cell growth status or a growth-independent action by insulin directly on IGF-1 receptor expression. To address these issues, IGF-1 receptor levels were monitored by Western blot analysis over a 48-h period following the addition of insulin to quiescent H4IIE cells. This approach was selected because the metabolic conversion from a gluconeogenic to a glycogenic phenotype occurs in less than 6 h, as indicated by the a reduction in PEPCK mRNA levels, whereas approximately 12 h is required to reach S phase [10]. These experiments showed that IGF-1 receptor levels remain unchanged over the first 6 h, but they increased approximately 4-fold by 12 h (Fig. 8). This increase is transient, however, since receptor levels peak between 12 and 24 h and subsequently decrease.

4. Discussion

Although the liver is the primary source for circulating IGF-1, it remains unresolved whether IGF-1 influences hepatic function directly. This uncertainty arises from contradictory evidence regarding the existence of IGF-1 receptors in the liver [21]. Also, it has been established that insulin mediates hepatic regeneration following injury [3]. Thus the distinction typically observed in other tissues, where insulin operates as a hormone to control cellular metabolism while IGF-1 functions as a mitogen, is not apparent in the liver.

Hepatic IGF-1 receptor density is highest in fetal tissue [5,24]. In the adult liver, however, IGF-1 receptors have been detected by some investigators [5,21], but not by others [2,4,6]. These conflicting results may represent differences in sensitivity related to a specific methodology, the source of the material used for the experiments (i.e. intact cells or membranes) or the animal species, and some of these issues have been addressed by McElduff et al. [21]. It is particularly noteworthy that IGF-1 receptors were near the limits of detection even in those studies demonstrating their presence [5] and an RNase protection study has confirmed that IGF-1 receptor mRNA levels in adult liver are 94% lower than in the fetal tissue [24]. On the other hand, it is evident that IGF-1 receptor density increases significantly in regenerating liver [5]. Correspondingly high levels of IGF-1 receptors have also been observed on hepatoma cells which typically exhibit high rates of proliferation [20,25,26]. Thus the developmental and growth state of the tissue is an important factor in IGF-1 receptor expression.

While IGF-1 apparently does not stimulate hepatocyte proliferation following hepatectomy, it may influence a restricted set of metabolic pathways associated with glucose utilization in the liver [25,27,28]. These studies, however, were conducted with hepatoma cell lines, and their properties may not resemble those of hepatocytes in many ways. For example, only the H-35 and KRC7 hepatomas have been reported to lack IGF-1 receptors and in this way resemble adult hepatocytes more closely [12,13,27]. In contrast, other hepatoma lines, including the commonly used HepG2, BRL 3A and HTC hepatomas, have an abundance of IGF-1 receptors [20,25,26]. The H4IIE hepatoma line has been well characterized with respect to insulin [8,23,29], but the response of these cells to IGF-1 has received limited attention. Our interest in this aspect was prompted by an ob-

servation that IGF-1 induced certain growth-associated processes, but was unable to stimulate DNA synthesis ([11], Yau et al. manuscript in preparation). Since H4IIE cells were derived in conjunction with the Reuber H-35 hepatoma [8,30], it has been assumed that they would exhibit similar characteristics. In this study of H4IIE cells, however, we have demonstrated that H4IIE cells express receptors for IGF-1 (Figs. 1–4). Although the observed 175 kDa molecular mass is higher than the more accepted size of 135 kDa, the ability of IGF-1, but not insulin or IGF-II, to effectively compete for the ligand indicates specificity for IGF-1. Furthermore, the difference in apparent molecular mass can be accounted for by the degree of N-linked glycosylation (Fig. 3). Additionally, we have observed that receptor density varies with growth state (Fig. 5). Interestingly, while a similar correlation between growth state and IGF-1 receptor density has been noted in studies comparing fetal, adult and regenerating liver [5], the direction of this change is reversed with respect to our findings. Insulin receptor levels, on the other hand, have been shown to increase in serum-starved HepG2 cells [31]. While some of the observed changes in receptor expression may reflect the importance of IGF-1 to cell cycle progression in other systems, additional studies will be required to define the contribution of IGF-1 receptors to H4IIE cells.

Insulin functions as a negative regulator of insulin receptor expression [31,32] and there is considerable evidence that insulin may similarly control IGF-1 receptor expression [33]. For instance, IGF-1 receptors were found to increase during fasting [34] and in an experimental model of diabetes [35], and both conditions are associated with low circulating insulin concentrations. Furthermore, IGF-1 receptor levels in diabetic rats were normalized by insulin treatment [35]. Although our observations that IGF-1 receptor protein and mRNA quantity is increased in quiescent cells (Figs. 6 and 7) are diametrically opposite to the previously mentioned studies, they may nevertheless indicate that modulation of IGF-1 receptor expression is not a consequence of changes in growth state, but rather of direct control by insulin [36]. This issue was addressed by monitoring IGF-1 receptor levels after adding insulin to quiescent H4IIE cells and the increase in receptor amount (Fig. 8) clearly indicates that IGF-1 receptor expression is enhanced by insulin. Furthermore, no comparable change in insulin receptor levels was detected upon probing the same blots with an insulin receptor-specific antibody (data not shown). Therefore, the absence of insulin from the culture media used to encourage entry into a quiescent state may not be the primary mediator of the increase in IGF-1 receptor numbers and, while these observations do not conform with other published reports on this topic [36], they do agree with the findings of Hatada et al. [31] based on their study of insulin receptor regulation.

The results presented in this report indicate that IGF-1 receptors are present on H4IIE hepatoma cells and that these proteins are inversely regulated by growth state. Since insulin is the principal factor controlling the growth of these cells, insulin may also control IGF-1 gene expression directly. While a clear distinction between a proliferation-mediated and an insulin-mediated mechanism for the regulation of IGF-1 receptors cannot be made, our experiments suggest that these factors operate independently to influence IGF-1 receptor levels.

Acknowledgements

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