Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor

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Summary Insulin has traditionally been considered as a hormone essential for metabolic regulation, while the insulin-like growth factors (IGF-I and IGF-II) are postulated to be more specifically involved in growth regulation. The conventional wisdom is that they share each other's effects only at high concentrations, due to their weak affinity for the heterologous receptor. We discuss here the evidence that in the proper cellular context, insulin can be mitogenic at physiologic concentrations through its own receptor. We studied the insulin and IGF-I binding characteristics of a new model suitable for analysing insulin receptor mediated mitogenesis; that is, a T-cell lymphoma line that depends on insulin for growth, but is unresponsive to IGFs. The cells showed no specific binding of ^{125}I-IGF-I and furthermore, no IGF-I receptor mRNA was detected by RNAse protection assay in the LB cells, in contrast with mouse brain and thymus. The cells bound at saturation about 3000 insulin molecules to receptors that had normal characteristics in terms of affinity, kinetics, pH dependence and negative co-operativity. A series of insulin analogues competed for ^{125}I-insulin binding with relative potencies comparable to those observed in other insulin target cells. The full sequence of the insulin receptor cDNA was determined and found to be identical to the published sequence of the murine insulin receptor cDNA. The LB cell line is therefore an ideal model with which to investigate insulin mitogenic signalling without interference from the IGF-I receptor. Using this model, we have started approaching the molecular basis of insulin-induced mitogenesis, in particular the role of signalling kinetics in choosing between mitogenic and metabolic pathways. [Diabetologia (1997) 40: S 25–S 31]

Keywords Insulin receptor, IGF-I receptor, mitogenic signalling, insulin analogues, T-cell lymphoma.

The receptors for insulin and insulin-like factor-I (IGF-I) belong to the family of receptor protein tyrosine kinases [1]. Although a vast body of data supports the concept that insulin stimulates cell growth in vitro and in vivo, the question of whether

insulin is physiologically a growth factor remains controversial (for review see [2]). Even more controversial is the question of whether insulin is capable of inducing mitogenic effects through its own receptor, or whether the growth-promoting effects of insulin result from its weak interaction with the IGF-I receptor or occur within insulin/IGF-I receptor hybrids [3, 4], or via interphosphorylation of the IGF-I receptor by the insulin receptor tyrosine kinase [5]. The response possibly depends on the cell type and its given supply of insulin and IGF-I receptors as well as the subsets of intracellular signalling molecules that are activated by either receptor. (We use the term IGF-I receptor for simplicity to designate the type 1 IGF receptor which binds both IGF-I and II and probably mediates the mitogenic effects of both growth factors [6].)

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Abbreviations: MAP kinase, Mitogen-activated protein kinase; MES, 2-[N-Morpholino]ethanesulphonic acid; ACES, 2-[(2-Amino-2-oxoethyl)amino]ethanesulphonic acid; EPPS, N-[2-Hydroxyethyl]piperazine-N′-[3-propanesulphonic acid]; TAPS, N-tris[Hydroxymethyl]methyl-3-aminopropanesulphonic acid; IGF-I, insulin-like growth factor-I; CHO, Chinese hamster ovary cell; HEPES, 4-[2-hydroxyethyl]-1-piperazineethanesulphonic acid; IL, interleukin; PI, phosphatidylinositol.

In this brief review, we will summarize the evidence supporting the concept that the insulin receptor is fully capable of transmitting mitogenic signals, and describe a novel cell model suitable for studying such signalling by insulin.

Previous evidence that the insulin receptor is mitogenic

It is usually assumed that insulin is primarily a metabolic hormone while IGF-I is primarily a growth factor, but that they share each other's properties at high concentrations due to the weak affinity of each ligand for the other receptor [2, 7, 8].

The fact that many cells contain both types of receptor complicates the assignment of the mitogenic effect to either receptor. When the stimulation of thymidine incorporation in a given cell type by insulin gives a poorly sensitive curve, it is usually assumed that the response is mediated through the IGF-I receptor. In a key experiment, Ursø and Gammeltoft (personal communication) have shown that by transfecting NIH3T3 cells, which show a poor sensitivity to insulin, with increasing amounts of insulin receptor cDNA, the insulin dose-response curve for thymidine incorporation shifts toward lower concentrations when the cells express more insulin receptors than IGF-I receptors. These data suggest that the response now goes through the insulin receptor. In contrast, the IGF-I curve becomes less sensitive. It has been suggested that when co-expressed, the receptor present in excess inhibits the other's mitogenic potential, either through hybrid receptor formation or by competition for intracellular substrates [8, 9].

Other experimental evidence suggests that the insulin receptor tyrosine kinase has oncogenic potential. Wang et al. [10] showed that a chimeric retrovirus (UIR) with the *ros* sequence of the avian sarcoma virus UR2 with human insulin receptor cDNA sequences coding for 46 residues of the extracellular domain and the entire transmembrane and cytoplasmic domains of the insulin receptor β subunit was capable of transforming chicken embryo fibroblasts and promoting formation of colonies in soft agar. A variant that arose from the parental UIR efficiently induced sarcomas in vivo in chickens (Fig. 1).

While this demonstration of the oncogenic potential of a constitutively activated insulin receptor tyrosine kinase was performed in a clearly artificial system, recent data with insulin analogues have also raised concerns about the potential carcinogenicity of some modified insulins, and the need for accurate tests thereof. The fast acting (in vivo) insulin analogue Asp^{B10} insulin was indeed found to induce both benign (fibroepithelial adenomas) and malignant (adenocarcinomas) mammary tumours in female rats [11]. It was also found to be supermitogenic

Fig. 1. Induction of sarcoma in chicken by chick embryo fibroblasts transformed by a constitutively activated insulin receptor tyrosine kinase. Reproduced with permission from [45]. Figure kindly provided by W.J. Rutter

in vitro in that its potency to stimulate thymidine incorporation in vascular smooth muscle cells was 10–20 times higher than expected from its affinity for the insulin receptor [12]. We [13] and others [14] found that this enhanced mitogenicity is a general property of analogues with a prolonged residence time on the insulin receptor relative to native insulin, due to a slower dissociation rate, as discussed below.

Finally, there is some circumstantial evidence that chronic hyperinsulinaemia may be correlated with breast cancer risk in humans [15].

Cell models suitable for investigating insulin's mitogenic effects

To the best of our knowledge, only hepatoma cell lines such as H35 or KRC7 have been shown to lack IGF-I binding and to still exhibit sensitive insulin-dependent growth and thymidine incorporation [16–18]. A few other cell lines (such as the F9 embryonal carcinoma cell line [19] and RPMI-8226 [20]) show sensitive

insulin mitogenic responses but the status of IGF-I receptors in these cells has not been established. Mamounas et al. [21, 22] have established an interesting insulin-dependent Chinese hamster ovary cell line (CHO-K1) where insulin appears to be mitogenic through its own receptor; however, this cell line also binds IGF-I. In no case has the status of IGF-I receptors been ascertained by methods other than ligand binding.

Lammers et al. [23] have used NIH3T3 cells overexpressing chimeric insulin and IGF-I receptor constructs in order to compare the intrinsic mitogenic potency of their respective intracellular domains, and found only a 10-fold difference between the potencies of the insulin and IGF-I receptor domains. There is however a general problem with using cellular overexpression of receptors or signalling molecules, in that the high level of expression may overcome low affinities and promote interactions that would not occur under more physiological conditions.

We have recently explored a novel in vitro model, the LB cell lymphoma line, well suited to the investigation of the mitogenic signalling through the insulin receptors [24].

The LB T lymphoma line

The LB cell line is a murine T-cell lymphoma designated LB that demonstrates insulin-dependent growth. This very invasive tumour [25], initially qualified as a lymphoid T-cell leukemia, arose spontaneously in a 6-month-old BALB/c mouse in the animal colony of the Academia Nacional de Medicina in Buenos Aires [26, 27]. It was noticed that the cells grew much better in medium containing insulin than in medium lacking it [28]. Thymidine incorporation into LB cells (as well as cell number) was markedly stimulated over a wide range of insulin concentrations (including low physiological), but produced only a negligible response to IGF-I and none to IGF-II [28]. The cell proliferation was blocked by anti-insulin antibodies. In a previous characterization, the cells were shown to bind ^{125}I-insulin and to contain around 3200 high affinity insulin binding sites [28]. Furthermore, insulin deficiency generated by a low energy diet or streptozotocin-induced insulin-dependent diabetes produced resistance to lymphoma growth in mice [29], demonstrating insulin dependence in vivo.

Characteristics of the insulin receptor on the LB cells

Growth characteristics in vitro. Although the LB cells could be entirely grown in vitro in suspension in complete RPMI 1640 medium containing insulin, their doubling time was slow (4 days, vs e.g. 1 day for EBV-transformed human lymphocytes [unpublished
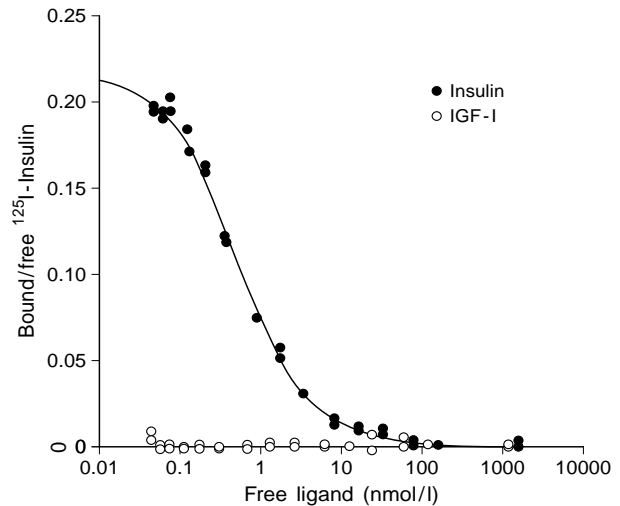

Fig. 2. Competition curves for binding of ^{125}I-insulin and ^{125}I-IGF-I to LB cells. ^{125}I-insulin or ^{125}I-IGF-I (2×10^{-11} mol/l) was incubated in binding buffer with $3–5 \times 10^7$ LB cells for 4 h at $4°C$ in the presence of increasing concentrations of unlabelled ligand (0–1.7 µmol/l) or analogues at appropriately scaled concentrations. At the end of incubation, duplicate 200 µl aliquots were placed in Eppendorf Microfuge tubes containing 200 µl prechilled buffer and centrifuged at 10 000 *g* for 5 min. The supernatants were aspirated and the pellets counted in a gamma counter with 80 % efficiency. The radioactivity bound to the cells in the presence of 1.7 µmol/l unlabelled ligand was subtracted as non-specific binding. Specific binding (as bound/free ligand) is plotted as a function of free ligand concentration. The competition data were analysed on a Sun workstation using non-linear curve-fitting programs developed at the Hagedorn Research Institute. A negative co-operativity model assuming one high-affinity and one low affinity bound ligand per receptor heterotetramer [7] was used to fit the data. The model was expressed as a two-site Adair model [43] with dissociation constant K_{d1} different from K_{d2} but receptor site concentration $R_1 = R_2$

observations]). This slow growth rate in vitro made it more feasible, in order to obtain large numbers of cells, to maintain them by serial passage in the peritoneal cavity of syngeneic mice [26]. One half millilitre of cell suspension (2×10^6 cells/ml RPMI) was injected into the peritoneal cavity of 6-week-old female BALB/c mice. One week later, ascites was removed. The cells were washed three times in HEPES (4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid) binding buffer before further studies.

Insulin and IGF-I binding. Preliminary experiments (not shown) determined that steady-state ^{125}I-insulin binding was reached after 3 h at $4°C$. The curve for competition of ^{125}I-insulin by increasing amounts of unlabelled insulin (Fig. 2) showed high affinity binding ($IC_{50} \sim 0.5$ nmol/l). In contrast, no specific binding of ^{125}I-IGF-I could be demonstrated; there was only 0.1 % of non-specific binding (Fig. 2).

RNAse protection assay of IGF-I receptor mRNA. The absence of ^{125}I-IGF-I binding sites on the LB
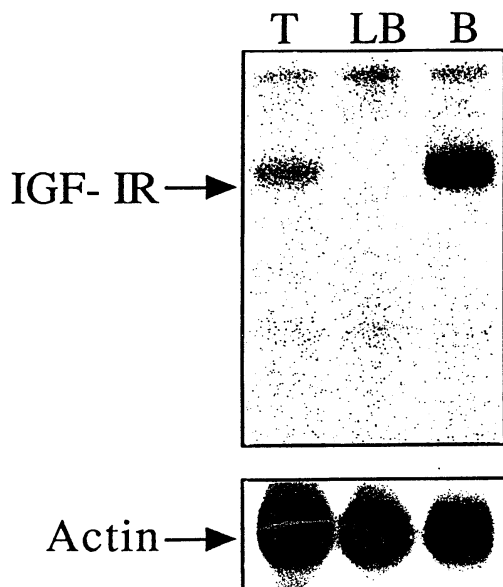
T   LB   B

IGF- IR →

Actin →

Fig. 3. RNAse protection assay of IGF-I receptor mRNA. *Left lane*: RNA from BALB/c mouse thymus. *Middle lane:* RNA from LB cells. *Right lane*: RNA from BALB/c mouse brain. *Top*: IGF-I receptor probe. *Bottom*: β-actin probe. Solution hybridization/RNAse protection assay was performed as previously described [44], with slight modification. Briefly, 20 μg of total RNA was hybridized with 1×10^6 cpm of ^{32}P-labelled antisense RNA probe of IGF-I receptor (or β-actin probe). Hybridization was performed in a buffer containing 75% formamide at 42 °C for 16 h. After hybridization, RNA samples were digested with RNAse A at 2 μg/ml and RNAse T1 at 1.1 μg/ml for 15 min at 30 °C, ethanol-precipitated and electrophoresed on an 8% polyacrylamide/8 mol/l urea denaturing gel. The gel was dried and exposed to X-ray film at –70 °C using an intensifying screen. The plasmid used for preparation of ^{32}P-labelled rat IGF-I receptor antisense RNA was a gift from Dr. D. LeRoith (Diabetes Branch, National Institutes of Health, Bethesda, MD., USA)

Fig. 4. Scatchard plot of insulin binding to LB cells. The computer-fitted insulin competition data of Figure 2 are displayed as bound/free vs bound insulin

Fig. 5. PH dependence of ^{125}I-insulin binding to LB cells. ^{125}I-insulin (1.7×10^{-11} mol/l) was incubated with LB cells (5×10^7/ ml) in the absence or presence of 1.7 μmol/l unlabelled insulin (non-specific binding) in 500 μl binding buffer adjusted to the appropriate pH. The buffer composition was the same as above except that the following buffering agents were used (100 mmol/l): pH 5.5–6: 2-[N-morpholino]ethanesulphonic acid (MES); pH 6.2–6.8: 2-[(2-Amino-2-oxoethyl)amino]ethane sulphonic acid] (ACES); pH 7.2–7.6: HEPES; pH 7.4–7.8: N-[2-Hydroxyethyl]piperazine-N′-[3-propane sulphonic acid] [EPPS]; pH 8–9: N-tris[Hyroxymethyl]methyl-3-aminosulphonic acid [TAPS]; pH 10: glycine (all from Sigma, St. Louis, MO, USA). After 4 h at 4°C, duplicate 200 μl from each tube were centrifuged and processed as described in the legend of Figure 2

cells was confirmed by RNAse protection assay (Fig. 3).

The presence of mRNA for the IGF-I receptor was demonstrated in the brain and thymus of BALB/c mice using a probe for the rat IGF-I receptor; in contrast, no IGF-I mRNA was detected in the same amount of total RNA from LB cells. Similar amounts of actin mRNA as control were detected in all three samples.

Properties of the insulin receptor on LB cells. Since overexpression of insulin receptors or expression of an abnormal insulin receptor on LB cells may be associated with the malignant state, we undertook a detailed characterisation of the insulin receptor on these cells.

The insulin equilibrium binding data showed excellent fit (Figs. 2 and 4) to a model of negative co-operativity assuming one molecule of ligand bound with high affinity ($K_{d1} = 0.88$ nmol/l) and one with low affinity ($K_{d2} = 5.5$ nmol/l) per receptor
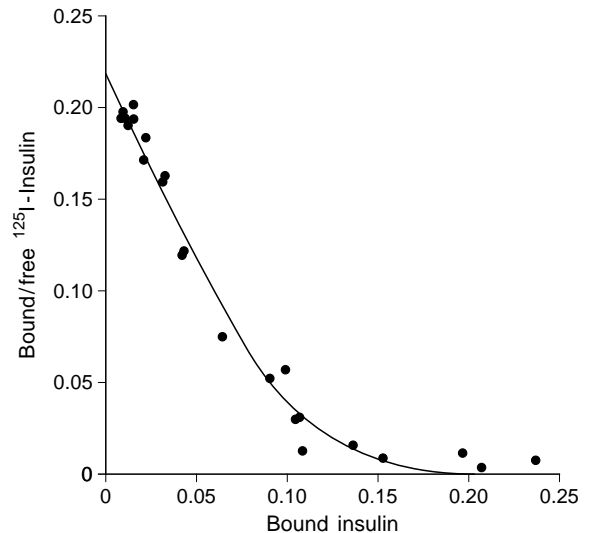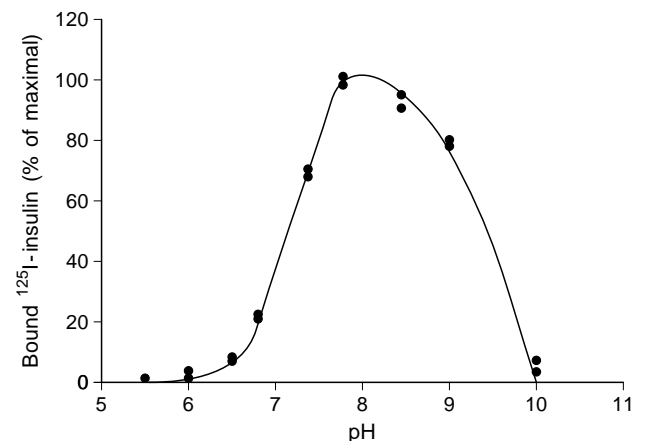
heterotetramer, a model previously shown to apply to insulin as well as IGF-I binding to multiple cell types [7, 30]. The total number of binding sites was only 3000, corresponding to 1500 receptors per cell assuming half of the sites were reactive [7].

The pH dependence of binding showed a bell-shaped curve with a maximum at pH 7.6 (Fig. 5) as
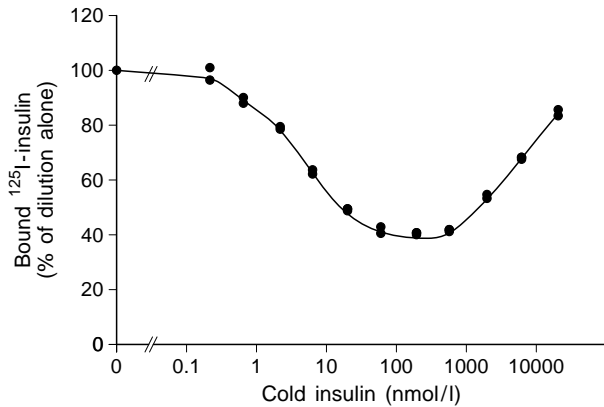
Fig. 6. Dose-response curve for negative co-operativity of the LB cell insulin receptor. The ability of unlabelled insulin to accelerate the dissociation rate of ^{125}I- insulin at high dilution, the hallmark of negative co-operativity [7, 30, 31] was studied as a function of unlabelled insulin concentration in the dilution medium as previously described [31]. Briefly, 10^{-10} mol/l ^{125}I-insulin was incubated with 3×10^{8} LB cells in 1 ml buffer at 4°C for 3 h and then centrifuged. The supernatant was aspirated and the pellet was resuspended in 1 ml ice-cold buffer, from which 25 μl aliquots were quickly transferred into duplicate tubes containing 1 ml binding buffer (4°C) with increasing concentrations of unlabelled insulin (0–8 μmol/l). The dissociation was allowed to proceed for 30 min, after which the tubes were centrifuged and the pellets counted
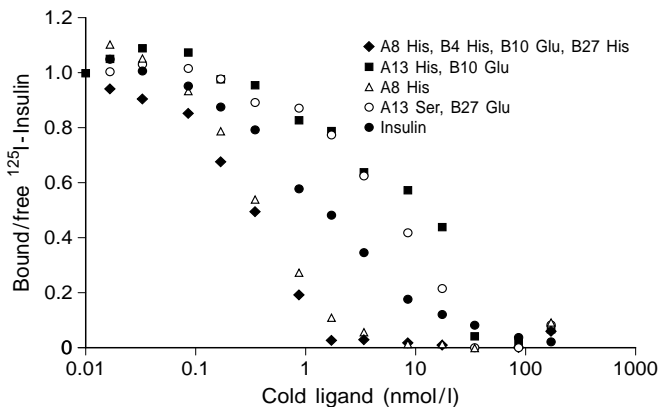

Fig. 7. Competition of insulin analogues for ^{125}I-insulin binding to LB cells. ^{125}I-insulin was incubated with LB cells in the presence of various insulin analogues at the indicated concentrations. Specific tracer binding is plotted vs analogue concentration

previously found for the insulin receptor on other target cells [31].

Insulin dissociation kinetics displayed typical features of negative co-operativity [30, 32, 33]. The dissociation rate of ^{125}I-insulin was accelerated by unlabelled insulin with a bell-shaped dose-response curve identical to that previously described in various cell types (Fig. 6), with a maximum effect obtained with 100 nmol/l unlabelled insulin.

A number of insulin analogues (four are shown in Fig. 7) competed for ^{125}I-insulin binding with relative

potencies comparable to those shown previously for the insulin receptor in HepG2 cells [34, 35] and to their biological potency in the free fat cell assay [34, 35]. Collectively, these results indicate that the LB cell insulin receptor does not exhibit exceptional properties as compared to other insulin receptors. To further establish this conclusion, the cDNA sequence of the LB insulin receptor was analysed.

Sequence of the LB cell insulin receptor cDNA. The LB cell insulin receptor cDNA sequence was identical to the published mouse sequence [36] with the exception of two silent single base changes. Since we found the same variations in the control liver cDNA from BALB/c mice, these variations most likely represent sequencing errors in the published sequence or allelic polymorphisms. Moreover, we found a C at bp position 3314 which is in agreement with the published sequence [36] but in disagreement with the database sequence which has T at this position (accession nr.: J05149.GB RO; GenEMBL), which is probably an error since again we found the same variation in BALB/c mouse liver cDNA. The change from T to C would change the corresponding amino acid (at position 1062) from methionine to threonine.

In summary, we have clearly documented by both ligand binding studies and RNAse protection assay the complete absence of IGF-I receptors in LB cells. Therefore, the observed mitogenic response is clearly mediated through the insulin receptor. Unlike a number of mutant insulin receptors [37], the small number (1500) of LB cell insulin receptors present have the normal binding characteristics of the insulin receptor previously described on other target cells: affinity, pH dependence, negative co-operativity and relative affinity for insulin analogues. Moreover, the sequence of the receptor cDNA was essentially identical to the published murine sequence, with the exception of two residue differences also present in the liver of control BALB/c mice and therefore likely to represent sequencing errors in the published sequence or allelic variants.

The presence of insulin receptors and the absence of IGF-I receptors on a T-cell line is not a common phenomenon, although it has only been systematically investigated in human cell lines. Typically, most human T-cell lines will have IGF-I but not insulin receptors, while the reverse is true for B-cell lines [38, 39]. Lee et al. [38] suggested that IGF-I and insulin receptors may be markers of lymphocyte differentiation and that these peptide growth factors may play a role in lymphocyte metabolism.

Besides insulin, LB cell proliferation is also stimulated by interleukin (IL)-2 [28], IL-4 (Ursø, B., unpublished data), γ-interferon, concanavalin A, and phorbol 12-myristate 13-acetate (PMA) (Naor, D., unpublished data), and inhibited by tumour necrosis factor α in the presence of serum (Ursø, B., unpublished

data). Recently, we found that human growth hormone is also a powerful mitogen for the LB cells (Ilondo, M. M. et al, manuscript in preparation). In contrast, they do not respond to IL-I, platelet-derived growth factor or c-kit (Ursø, B., unpublished data). Altogether, these findings suggest that LB cells exploit, in addition to insulin, other growth signals, which may collaborate with insulin or exert an independent stimulus.

The nature of the oncogene(s) responsible for the malignancy and invasiveness of the LB lymphoma is unclear. It is clearly not an abnormal insulin receptor, but one factor could be the lack of IGF-I receptors if the above-mentioned hypothesis that the two receptors keep each other's mitogenic potential in check is correct. It could also be that a downstream signalling molecule is mutated, constitutively activated or abnormally expressed, making it overresponsive to insulin or resistant to downregulation control mechanisms.

In any case the LB cells should be an interesting new model with which to investigate insulin mitogenic signalling without interference from the IGF-I receptor.

Mitogenic signalling pathway of insulin in the LB lymphoma cells

Our preliminary data indicate that both the Ras/Raf/MEK/MAP kinase pathways and the phosphatidylinositol (PI)-3 kinase pathways are active in the LB cells, but that mitogen activated protein (MAP) kinase is much less stimulated by insulin than PI-3 kinase, in line with much recent evidence in a variety of systems that tends to downplay the importance of MAP kinase in a number of insulin effects (reviewed in [40]). The effect of specific inhibitors of MAP kinase/extracellular signal regulated protein kinase (MEK) (PD-098059) and PI-3 kinase (LY24009), which partially inhibited the mitogenic response to insulin, was difficult to interpret since the inhibitors also inhibited at similar doses the basal level of thymidine incorporation in the absence of insulin, suggesting perhaps that a tonic basal level of both pathways is permissive for mitogenesis, but not allowing firm conclusions as to their role in insulin stimulation. We are also exploring, based on preliminary evidence, the possibility that the LB cells express an insulin substrate docking protein different from IRS-1 and IRS-2 (Ursø, B. et al., in preparation).

Conclusions: what is the molecular basis of the mitogenic compared to metabolic specificity of insulin and IGF-I?

It is obvious from the above brief survey that at this point we do not have a clear understanding of the molecular mechanisms that determine how insulin or IGF-I trigger mitogenic or metabolic effects through their own or each other's receptors in a given cell type. This problem is compounded by the fact that both ligands appear capable of stimulating the same network of signalling cascades in various cells (reviewed in [8, 41, 42]).

The finding that mitogenic effects are selectively enhanced by an increased residence time of insulin analogues on the receptor [13, 14], resulting, in CHO cells overexpressing the insulin receptor, in a sustained activation of the insulin receptor tyrosine kinase and phosphorylation of Shc [14], suggests that a detailed investigation of the kinetics of downstream signalling is required in order to fully understand the molecular basis of signalling specificity. The LB cell line will be a useful model with which to pursue this line of investigation.

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