NUCLEAR COLOCALIZATION AND COMPLEX FORMATION OF INSULIN WITH RETINOBLASTOMA PROTEIN IN HEPG2 HUMAN HEPATOMA CELLS

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ABSTRACT

Previous structural and biochemical evidence had suggested that insulin may bind to the nuclear tumor suppressor retinoblastoma protein (RB). The present study is now the first to unravel the physical and functional interaction between a growth factor and an anti-oncoprotein, specifically demonstrating the association between insulin and RB in living cells and finding that this complex formation is relevant for cell division. Our immunofluorescence microscopy data suggest that insulin colocalizes with RB in the cell nuclei of HepG2 human hepatoma cells and that it contacts the B-region of the RB pocket. Furthermore, these events were found to correlate with an enhancement of cell proliferation. These results are in line with the initial structure-based predictions and, moreover, provide a suitable starting point for the further understanding as well as the pharmacological modulation of nucleocrine interactions between growth factors and tumor suppressors, in physiology and disease.

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Introduction

Insulin is a major hormone in the circulation regulating glucose homeostasis and bioavailability, yet it is also an important growth factor for normal and neoplastic cells. Its action is still interpreted as to result solely from its interaction with its cell membrane receptor and the signaling cascades ensuing therefrom (1). By contrast, a previous analysis of insulin's amino acid sequence had already suggested that, intriguingly, insulin may also bind to the nuclear tumor suppressor RB which is known to play a central role in the control of cell cycle progression (2). Subsequently, the RB peptide proposed to be the contact site for insulin was shown to recognize full-length human insulin *in vitro* (3). Based on these studies, we addressed the question whether insulin could also be found to associate with RB in living cells. We chose as a model the HepG2 human hepatoma cells which have already been shown to contain normal RB (4). We now report that insulin translocates to the nuclei of these cells where it associates with RB, more specifically with the B-region of the RB pocket, and that this complex formation coincides with an increase in cell division.

Materials and Methods

Cell Lines and Tissue Culture. HepG2 cells (ATCC), a human hepatocellular carcinoma cell line, were grown in RPMI medium (Life Technologies) supplemented with 5% FCS (Biochrom).

Internalization Experiments and Immunofluorescence

Microscopy. HepG2 cells were seeded on cover slips, allowed to adhere and subsequently incubated without serum for about 48 hr. The cells were then treated with bovine insulin (Sigma) at 100 µg/ml RPMI for 30–60 min at 37°C. Following this treatment, cells were processed according to a previously described protocol (5). Briefly, cells were fixed with 2% paraformaldehyde for 30 min at 4°C and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Cells were then incubated with primary antibodies for 90 min and subsequently with fluorescently labeled secondary antibodies for 30 min. Finally, nuclei were counterstained with Hoechst 33342 dye (Serva Feinbiochemica) and mounted with ProLong antifade (Molecular Probes) for immunofluorescence microscopy. Antibodies and dilutions for the experiments were the following: mouse anti-human RB primary antibodies (Fig. 1) G3-245 (Pharmingen) which recognizes the RB epitope 332–344 1:100 and XZ91 (Dunn Labortechnik) which binds to the RB site 620–665 (6) 1:20, guinea pig anti-human insulin antibody A0564 (Dako) 1:20, Cy3-labeled goat anti-mouse secondary antibody (Amersham Pharmacia Biotech) 1:50 and Alexa-488-labeled goat anti-guinea pig secondary antibody (MoBiTec) 1:100. Fluorescein-isothiocyanate (FITC)-labeled MCR-10 peptide (7) was a gift from Pichem, Inc. (Graz, Austria) and employed at a 50 µmolar concentration. Stained cells were examined using an Axio-

Fig. 1 Main RB domains, RB epitopes for anti-RB Abs G3-245 & XZ91 and proposed RB binding site for insulin

FIG. 2 Colocalization of insulin and RB in HepG2 nuclei whereby anti-RB Ab G3-245 generates a strong nuclear RB signal. (a) Hoechst 33342, (b) anti-insulin Ab, (c) anti-RB Ab G3-245, and (d) overlay of b & c.

skop microscope (Zeiss) with a x100 objective. Images were captured using 400 ASA slide films (Kodak).

Cell Proliferation Assay. HepG2 cells were seeded at 100,000 cells/ml RPMI/5% FCS in a total volume of 3 ml in Petri dishes with grids, allowed to adhere and subsequently incubated without serum for about 48 hr. The cells were counted in 4 to 5 different visual fields per dish for a first time as a baseline, using an Axiovert 35M microscope (Zeiss) with a x32 objective. The cells were then treated in the presence of RPMI/5% FCS with bovine insulin (Sigma) at 100 µg/ml and/or 50 µM FITC-MCR-10-peptide for 3–4 days at 37°C vs. RPMI/5% FCS alone as a control. Subsequently, the cells were counted again in the same areas as mentioned above. This experiment was repeated twice.

Results

In a first series of experiments, we found that, following a 30-minute incubation of HepG2 cells with insulin at 100 µg/ml (17 µM), the hormone was present in the nuclei (Fig. 2a) of the majority of the cells (Fig. 2b). The fluorescence signal was also detected in the cytoplasm, yet the fluorescence intensity was clearly higher in the nuclear compartment, suggesting that the predominant quantity of insulin had translocated to the nucleus. No signal was evident with the fluorescently labeled secondary antibody alone (not shown). On the other hand, RB could be visualized by means of the anti-RB antibody G3-245 in most HepG2 nuclei, with only a faint staining in the cytoplasm (Fig. 2c). The overlay then yielded that insulin and RB colocalize in the nuclei of these cells (Fig. 2d).

In order to more conclusively determine whether this nuclear colocalization also involved a physical association between these two proteins, we

performed a second series of experiments whereby we explored whether insulin could compete with an anti-RB antibody, namely XZ91, for the same RB binding site. In other words, we addressed the question whether insulin added to the cells prior to the specific anti-RB antibody would be able to cause the masking of the RB epitope of this antibody (Fig. 1) which is located in a region spanning RB amino acids 620–665 (6) and thus includes RB_{649–654} (Fig. 1), the proposed RB binding site for insulin (2). Fig. 3c suggests that this is the case since the fluorescence signal elicited by XZ91 in the cell nuclei, as identified by the Hoechst stain in Fig. 3a, is considerably fainter than the G3-245 signal (Fig. 2c) which is the result of an interaction of the latter antibody with the N-terminus of RB where insulin is *not* predicted to bind. Fig. 3b shows that insulin has translocated to the nucleus in this setting too and Fig. 3d reveals the colocalization of RB and insulin by merging the anti-RB and anti-insulin images.

To further verify whether the fainter staining with XZ91 vs. G3-245 is indeed a result of the prior RB epitope masking by insulin, we slightly increased the time of incubation with insulin to 1 hour since extending the time of exposure of the cells to insulin has previously been shown to increase the amount of insulin accumulating in the nucleus (8). We also employed FITC-MCR-10 (7), a fluorescently labeled peptide containing the proposed RB binding site for insulin in competition studies. As such, we observed in this third series of experiments that insulin is able to extinguish the XZ91 signal (Fig. 4d vs. Fig. 4b) and that the nuclear RB peptide (Fig. 4f) reverses this event, yielding a strong XZ91 signal despite the presence of added insulin (Fig. 4g).

FIG. 3 Colocalization of insulin and RB in HepG2 nuclei whereby anti-RB Ab XZ91 yields only a weak nuclear RB signal. (a) Hoechst 33342, (b) anti-insulin Ab, (c) anti-RB Ab XZ91, and (d) overlay of b & c.

FIG. 4 1-hour incubation of HepG2 cells with insulin at 100 $\mu\text{g/ml}$ completely masks the XZ91 epitope on RB and this event can be reversed by FITC-MCR-10. Control: (a) Hoechst 33342 & (b) anti-RB Ab XZ91. Treatment with insulin at 100 $\mu\text{g/ml}$: (c) Hoechst 33342 & (d) anti-RB Ab XZ91. Combined treatment with 50 μM FITC-MCR-10 and insulin at 100 $\mu\text{g/ml}$: (e) Hoechst 33342, (f) nuclear FITC-MCR-10 & (g) anti-RB Ab XZ91.

To exclude that XZ91 has recognized FITC-MCR-10 instead of RB, we also preincubated prior to its addition to the cells this peptide with the XZ91 antibody in a cuvette for 1 hour. If recognition had occurred, the peptide would have been precipitated during this preincubation and thus prevented from translocating into the nucleus and keeping insulin from concealing the XZ91 epitope on RB. However, we detected that the peptide localized to the nucleus and that XZ91 generated a potent RB signal (not shown).

Finally, we looked into the biological effects of insulin at the concentration at which we had observed its nuclear translocation and binding to RB, i.e. 100 $\mu\text{g/ml}$. In one representative experiment, insulin was found to significantly augment HepG2 cell proliferation after 96 hours of co-incubation whereby the median relative cell number in the insulin-treated cell sample was 1.89 (reflecting an increase from 204 to 385 cells per visual field) compared to 1.34 (from 179 to 240 cells per visual field) in the control, thus meaning that insulin raised the cell number by about 40% over control values. This rise in proliferation consistently correlated with considerable morphological changes such as a larger cell size as well as a shift of the nucleus/cytoplasm ratio towards the nucleus and the appearance of prominent nucleoli (not shown). By contrast, FITC-MCR-10 reduced HepG2 proliferation despite the presence of added insulin, decreasing the cell number to a median relative value of 0.77 (from 176 to 135 cells per visual field), i.e. by about 40% below control values. This effect was accompanied by morphological changes towards cellular differentiation, yielding fibroblast-like spindle forms and neuronal shapes (not shown) similar to what is seen when

normal RB is added to certain tumor cells.

Discussion

Insulin has previously been found to translocate to the cell nucleus of living cells (9). Although this finding is compatible with the nucleocrine model of an insulin-RB complex (10), the proof for the occurrence of this interaction *in vivo* has still been pending. The current report is therefore a first step towards *in vivo* validation. As such, we show here by immunofluorescence microscopy that, at pharmacological concentrations (100 $\mu\text{g/ml}$ or 17 μM , respectively), insulin translocates into the nuclei of the majority of living HepG2 cells which are both known to express normal RB (4) and to respond to insulin metabolically (11). By the same method, we demonstrate the colocalization and complex formation of insulin with RB. We infer from our data that, during the latter process, insulin binds the B region of the RB pocket, as anticipated (2). This is suggested by both the abrogation of the XZ91 RB recognition signal by insulin and the reversal of this event- resulting in a bright XZ91 RB staining- by FITC-MCR-10 (7) which contains the RB₆₄₉₋₆₅₄ fragment previously proven to recognize insulin (3). In other words, FITC-MCR-10 has most likely competed with RB for insulin and, by binding insulin, prevented it from masking the XZ91 epitope on RB, thus allowing sufficient XZ91 to reach its target and generate a strong signal. In this context, the preincubation experiment suggested that XZ91 recognizes endogenous RB protein, and not added synthetic RB peptide.

In contrast, endogenous or added insulin is not able to extinguish or even diminish the immuno-

fluorescence based on the G3-245 anti-RB antibody. This is in accordance with the previous predictions (2) since G3-245 recognizes an N-terminal epitope on RB (amino acids 332-344) that is *not* anticipated to participate in insulin-RB complex formation.

Furthermore, we observed that an anti-insulin fluorescence signal was also present in the nuclei of (serum-deprived) HepG2 cells that had *not* been pre-incubated with insulin (not shown), suggesting that these subcellular structures contain endogenously produced insulin with presumably autocrine functions. However, this signal was overall weaker than that in the nuclei of the cells pretreated with insulin. Under these baseline conditions, i.e. where no insulin had been added, the XZ91 RB signal was again fainter than the G3-245 RB signal (not shown). This may be due to the selective disturbance of the binding of XZ91 to RB by both endogenous nuclear insulin and other cellular proteins known to associate with the RB pocket such as D-type cyclins.

We finally found that insulin, at the same concentration at which it visibly colocalizes with RB in the majority of HepG2 nuclei and interferes with XZ91 binding to RB, also increases the number of HepG2 cells and causes them to assume a morphology that is characteristic of proliferating cells, implying that the binding of insulin to RB has a stimulating effect on cell proliferation. This had been correctly deduced from the initial structural analysis of insulin and its comparison with RB-binding viral oncoproteins (2).

The insulin concentration employed here is high. This experimental condition appears nevertheless reasonable. It has previously been reported that insulin translocates to the cell nucleus in a dose-dependent fashion, and this rather occurs in dose ranges above the concentrations known to stimulate the insulin receptors on the cell membrane (8). Moreover, in insulin-producing tumors the amount of insulin should be much higher than the insulin concentration of around 10 nM which is sufficient to activate the insulin receptor tyrosine kinase and also than the insulin concentration used by us here. This view is supported by a previous investigation into the insulin content of insulinomas that unraveled high pathological concentrations of insulin (0.1-1 mM) in these neoplastic tissues (12).

Interactions between growth factors and other proteins in the nuclei of living cells, e.g. between FGF and casein kinase II (13) or IGF-1 and IGFBP-3 (14), have already been reported. However, the occurrence of an association between insulin and RB *in vivo*, as outlined here for the first time, should be particularly important since, as a result of RB's immediate link to the genes, such a complex may enable insulin to directly influence the cell's decision to divide or not.

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