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Stable plasma membrane expression of the soluble domain of the human insulin receptor in yeast

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Abstract The soluble cytoplasmic kinase domain of the human insulin receptor was N-terminally equipped with either an *N*-acetylation or a dual-acylation motif (MGC box, to allow myristoylation/palmitoylation) and expressed in yeast cells under the control of the inducible *CUP1* promoter. Although the cellular concentration was about the same in both instances (reflecting similar stability against proteolysis), only the myristoylated protein was capable of autophosphorylation to a significant extent and was active to phosphorylate endogenous yeast proteins at tyrosine residues in vivo. Cellular subfractionation showed that the insulin receptor was associated with plasma membranes, from where it was not extractable with high salt or alkali, but a significant fraction was also localized in the nuclear fraction. The myristoylated protein is absent from the cytoplasm. No effect of expression of either the acetylated or the myristoylated version on growth and respiration on various carbon sources was detected, suggesting a failure of the active insulin receptor kinase domain to couple to yeast (glucose) signalling cascades.

Key words: Heterologous expression; Human insulin receptor; Tyrosine phosphorylation; *Saccharomyces cerevisiae*

1. Introduction

Glycogen metabolism in yeast – as in vertebrates – is controlled by nutrients, e.g. glucose, at the transcriptional level and, in addition, via a phosphorylation/dephosphorylation mechanism of the two key enzymes, glycogen synthetase (Gsy2p) and glycogen phosphorylase [1–4]. In the presence of glucose, Gsy2p is inhibited by phosphorylation on three serine residues in the C-terminal domain and, conversely, phosphorylase is activated by phosphorylation [5,6]. Previously, we have described that in yeast under conditions of growth limitation, these glucose-dependent effects are significantly enhanced by the simultaneous presence of human insulin (hIns) at concentrations 10–100-fold above those effective in mammalian insulin-responsive cells [6–8]. In addition, the glucose effect on several other enzymes (cAMP-dependent protein kinase A and cAMP phosphodiesterase, protein phosphatase 2A and glycosylphosphatidylinositol-specific phospholipase C) was enhanced analogously by hIns [6,8], strictly in the same fashion as in vertebrate insulin-responsive cells [9–11]. The regulatory responses correlated well with the specific hIns binding to a 53 kDa yeast plasma membrane protein (to which it could be cross-linked chemically), and with its ligand-dependent Ser/Thr phosphorylation. We did not observe any responses or binding to the membrane protein of growth factors closely related to insulin, such as insulin-like or epidermal growth factors, or of certain point mutations of hIns [12,13]. Together, these observations suggest that yeast harbors some components of an evolutionarily conserved mechanism which induces the multitude of intracellular processes in response to the presence of glucose that are subsumed under the term 'glucose repression' via an insulin-related signalling molecule.

The genes encoding the presumptive ligand and receptor have not yet been uncovered by the yeast genome sequencing project. From this failure and from the striking difference in molecular mass and phosphorylation behavior to vertebrate insulin receptors it is evident that, if yeast has an insulin recognizing protein, its primary structure is poorly conserved. We examined whether the soluble cytoplasmic domain of the β-chain of hIns receptor (hInsR), which has been demonstrated to exhibit similar catalytic properties and to use the identical sites in autophosphorylation as the native holo-receptor [14–18], could couple to intracellular components of the yeast glucose signalling cascade. We reasoned that these downstream constituents might be conserved in evolution to a higher degree than the putative insulin-like ligand and its receptor. We expressed hInsR in yeast cells in enzymatically active and plasma membrane-associated form and analyzed its effects.

2. Materials and methods

2.1. Cloning and expression of hInsR in yeast cells

The complete cytoplasmic domain of the β-subunit of hInsR (amino acid positions 940–1355 of the short version [19,20]) was amplified from a cDNA clone using forward primers which additionally contained an *N*-acetylation motif (amino acid sequence: MSSST...) (InsRAc: 5′-CTAGTATAATGTCTTCATCTACTAGATCTAATG-CTAGCAACCTGAGAAAGAGGCAGCCAG-3′) or an *N*-myristoylation site (amino acid sequence: MGCTVSSQT...) (InsRMy: 5′-CTAGTATAATGGGTTGTACTGTATCTTCCCAAACTAGATCT-AATGCTAGCAACCTGAGAAAGAGGCAGCCAG-3′). The reverse primer was InsRrev: 5′-CATGGTTAGGAAGGATTGGAC-CG-3′. The two amplificates were each ligated to the *Spe*I and *Nco*I sites of pEX [21]. The *CUP1* promoter was inserted into the respective *Bam*HI and *Spe*I sites of pEX as a *Bam*HI/*Nhe*I fragment [22]. Promoter, InsR coding sequence and terminator were recloned as a *Bam*HI/*Hin*dIII fragment in either the 2μ-based vector YEp352 [23] or the ARS/CEN vector pFL38 [24]. Yeast (strain CEN.PK2-1C MAT**a** [25]) transformed with one of these or a control plasmid (YEp352 or pFL38) was grown on 3% YPD medium overnight and induced with 1 mM $CuSO_4$ for 4 h.

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2.2. Cellular subfractionation

Yeast spheroplasts (from 4 l yeast culture, titer 2×10^7) were broken by shearing and osmotic lysis (0°C) in 0.6 M mannitol buffer containing a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.6 μg/ml pepstatin, 0.25 U/ml aprotinin, 10 mM sodium fluoride, 1 mM Na vanadate). The supernatant of a low speed pellet (5 min, $1500\times g$) was centrifuged (10 min, $20\,000\times g$) to obtain crude mitochondria and nuclei. This supernatant was then centrifuged (Beckman TLA-100.4 rotor, 45 min, $120\,000\times g$) to yield a particulate fraction, which was essentially free of mitochondria and nuclei and mainly contained plasma membranes, and a non-particulate cytoplasmic supernatant fraction. Crude mitochondria and nuclei were separated by centrifugation (30 min, $38\,000\times g$) in a 28% Percoll (Pharmacia, Freiburg) gradient [26]. All fractions were separated by SDS–PAGE (10%), electroblotted onto Immobilon P membrane (Millipore, Eschborn) and immunodecorated. Polyclonal anti-InsR (Santa Cruz, Heidelberg) and a mixture of anti-P-Tyr antisera (rabbit, purchased from Transduction Laboratories, Lexington, KY, and monoclonal PY99 from mouse, Santa Cruz, Heidelberg) or anti-peptide antibodies (goat, obtained from Santa Cruz, Heidelberg) directed against the marker proteins Rap1p (nuclei), Cyr1p (Cdc35p, plasma membranes and cytoplasm) or Cdc24p (plasma membranes) were used.

2.3. Miscellaneous procedures

Cell extracts used in Western blot analysis were prepared from 5 ml of a culture of logarithmically growing cells. The cell sediment plus 120 μl breakage buffer (20 mM Tris–HCl, pH 7.4, 2% SDS, 30 mM β-mercaptoethanol) plus 150 μl glass beads (0.45 mm in diameter) was boiled for 2 min and then homogenized on a Vortex for a total period of 2 min. The supernatant was directly used for SDS–PAGE. Extractions of subcellular fractions (100 μg of protein each) with high salt (1 M NaCl) or alkali (0.2 M Na_2CO_3, pH 10.5) was performed as previously described [27]. The soluble supernatant fraction and the pellet were analyzed separately by SDS–PAGE. Tyrosine-specific recombinant YOP phosphatase (from *Yersinia enterocolitica*) was purchased from New England Biolabs, Frankfurt. Phosphotyrosine was dephosphorylated as recommended by the manufacturer. Cells were broken with glass beads (10 pulses of 30 s each interrupted by cooling on ice, microscopic monitoring of breakage efficiency) in the presence of a protease inhibitor mix (end concentrations: 0. 25 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1.8 μg/ml pepstatin, 1.8 μg/ml leupeptin), and in the simultaneous presence or absence of 50 U YOP and/or phosphatase inhibitors (end concentrations: 1 mM (in the presence of YOP, 10 mM) $NaVO_3$, 10 mM NaF, 10 mM inorganic pyrophosphate, 10 mM α-glycerophosphate). The reactions were terminated by boiling in SDS buffer at the time points indicated and 50 μg protein per lane was separated by SDS–PAGE (10% gels) and immunodecorated. Protein determinations [28] and molecular procedures [29] have been described.

3. Results

The *CUP1* promoter was used for regulated expression of the human insulin receptor (hInsR) in yeast cells as its induction does not interfere with nutritional signalling (in contrast to the *GAL1/10* or the *PHO5* promoters). Expression from multi-copy plasmids of the two hInsR constructs was induced 3–10-fold by $CuSO_4$ (Fig. 1A). The cellular concentrations of the two different N-terminally modified versions were comparable. The electrophoretic mobilities of the recombinant proteins in SDS–PAGE corresponded to molecular masses of about 48 kDa. No degradation products were observed (in contrast to expression in *Escherichia coli*, not shown). In yeast strains defective for the proteasome subunits Pre1p and Pre2p [30] or for the vacuolar proteinases A and B and carboxypeptidases Y and S [31], the electrophoretic mobility was unchanged (not shown) indicating that the hInsR protein was proteolytically stable in vivo in the genetic background of the protease-proficient wild-type.

In order to protect the heterologous protein against possible

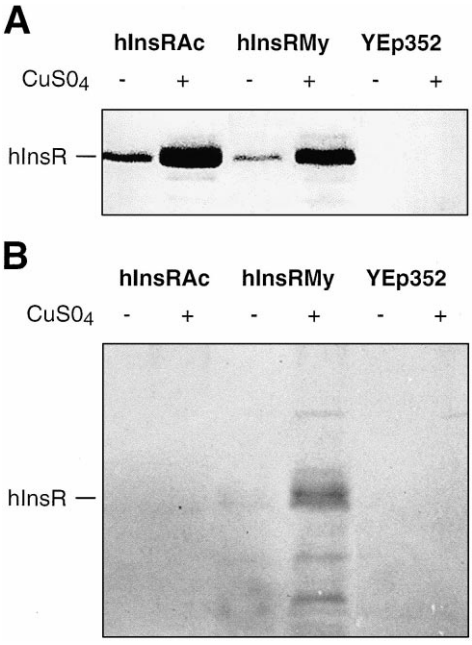

Fig. 1. Heterologous expression and tyrosine phosphorylation of the soluble domain of the human insulin receptor, hInsR. A: Induction of expression. Cells of the transformant strains harboring hInsRAc, hInsRMy or YEp352 (control) were grown in the absence or presence of 1 mM $CuSO_4$ (as indicated) for 4 h. The proteins were separated by SDS–PAGE, transferred to Immobilon P membrane and immunodecorated with a polyclonal anti-hInsR antiserum. B: Test for autophosphorylation of hInsR on tyrosine residues. The same protein preparations of transformant strains were used as in A and the Western blot decorated with a mixture of polyclonal anti-tyrosine phosphate antiserum and a monoclonal anti-P-Tyr antibody.

proteolytic degradation in vivo the two constructs had been equipped either with an *N*-acetylation sequence (deduced from the N-terminus of *N*-acetylated major adenylate kinase [32]), hInsRAc, or with an MGC box motif, MGCTVSS, hInsRMy, which was derived as a consensus from hSrc and the two yeast proteins, Gpa1p (which has been shown to be *N*-myristoylated in vivo [33]) and Gpa2p. In addition to mere protection from proteolysis, myristoylation of the N-terminal Gly in the mature protein and possible palmitoyl thioesterification of the neighboring cysteine residue was intended to allow association with the inner face of the plasma membrane. This was meant to mimic the topological situation in vertebrate cells. In mammalian insulin-responsive cells, insulin binding to the membrane-expressed dimeric receptor triggers a close juxtapositioning of the two β-chains of the holo-receptor to induce trans-chain autophosphorylation of the cytoplasmic domains [34,35]. In order to facilitate these bimolecular interactions within the insulin receptor and between the receptor and putative substrate proteins in yeast, the receptor construct comprised the complete juxtamembrane domain equipped with a membrane anchor in addition to the kinase and the C-terminal regulatory domains.

To examine whether the heterologously expressed InsR protein fragment exhibited enzymatic activity and was autophosphorylated in yeast, the same samples from glucose-grown cells were electrophoretically separated as in Fig. 1A but decorated with a mixture of polyclonal anti-P-Tyr antiserum and monoclonal PY99. A number of tyrosine-phosphorylated yeast proteins were detected in the transformant expressing

hInsRMy (Fig. 1B, lanes 3 and 4) whereas tyrosine phosphorylation was poorly to not detectable with the *N*-acetylated protein (lanes 1 and 2), although the cellular concentrations of the two versions of hInsR had been comparable (see Fig. 1A). One of the major signals in lanes 3 and 4 (corresponding to the lower band of the doublet indicated at the margin of the figure) coincided with hInsRMy showing that the protein was autophosphorylated. In addition, several signals of Tyr phosphorylation, corresponding to polypeptides, both larger and smaller than InsR, were found. Degradation of hInsRMy and unspecific cross-reactivity with non-phosphorylated proteins could be excluded by comparison with the polyclonal anti-hInsR antiserum or with the vector control, YEp352, respectively. Consequently these signals represent tyrosine-phosphorylated yeast proteins. Furthermore, phosphorylation of yeast proteins relied on the presence of autophosphorylated hInsRMy as it was absent from the controls (YEp352 vector, lanes 5 and 6 and from hInsRAc, lanes 1 and 2). The degree of Tyr phosphorylation of yeast proteins correlated with the strength of hInsRMy expression (compare non-induced and induced expression of hInsMy in lanes 3 and 4 of Fig. 1A,B). Similar signals were found after growth of cells on non-fermentable carbon sources (not shown).

To substantiate further that hInsRMy was phosphorylated on tyrosine residues, cells were broken after induction and proteins exposed to either endogenous phosphatases for increasing periods of time or to recombinant YOP tyrosine-specific protein phosphatase from *Y. enterocolitica*. As a control, cells were broken and incubated in the presence of a phosphatase inhibitor mix (vanadate, fluoride, α-glycerophosphate, inorganic pyrophosphate) which inhibits protein tyrosine phosphatases. Cell homogenates decorated with anti-hInsR antibody (Fig. 2A) served as the loading control. The phosphate residues were rapidly removed by added tyrosine phosphatase (YOP) (Fig. 2B, lane 10) as well as by endogenous yeast tyrosine phosphatases (lanes 1–4) as evident from the disappearance of the signal after incubation with the mixture of anti-P-Tyr-specific antisera. On the other hand, phosphotyrosine was protected to a significant extent by the inhibitor mix (lanes 5–8) which is particularly significant in the case of simultaneous presence of YOP and inhibitors (lane 9) to demonstrate P-Tyr specificity of YOP and its inhibition by the mix.

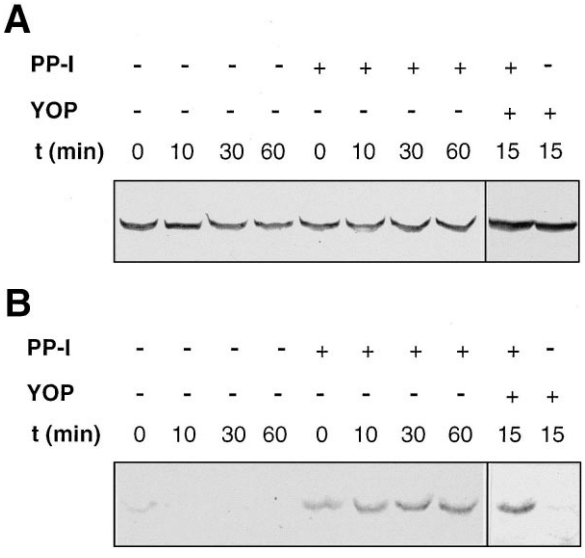

Fig. 2. Treatment with protein tyrosine phosphatase. Proteins from the hInsRMy-containing transformant strain were prepared in the presence of a mix of protease inhibitors with or without additional protein tyrosine phosphatase inhibitors (PP-I) or P-Tyr phosphatase (YOP) as indicated. 40 μg of protein was incubated at room temperature in the absence or presence of YOP P-tyrosine phosphatase (50 U) for the periods indicated t (min), separated by SDS–PAGE and immunodecorated with antisera directed against hInsR (A) or P-Tyr (B).

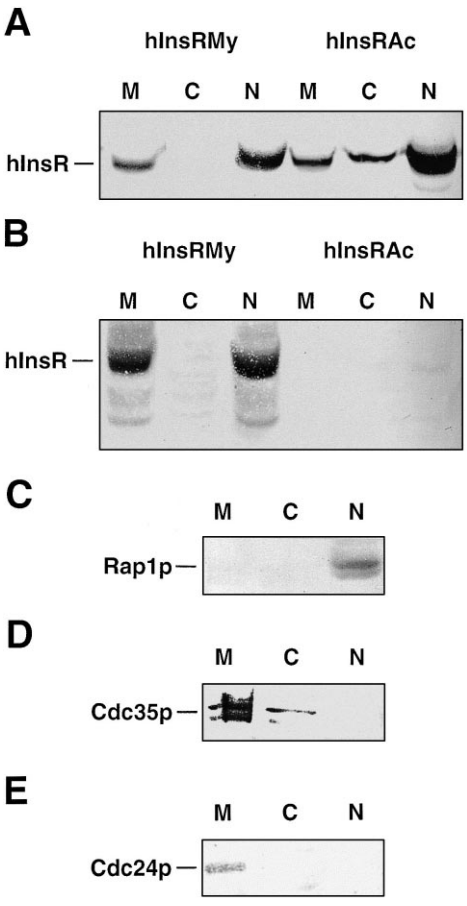

Fig. 3. Subfractionation of hInsRAc and hInsRMy transformant cells. Nuclei (N), plasma membranes (M) and soluble cytoplasmic fraction (C) from hInsRMy- or hInsRAc-expressing transformants were separated by SDS–PAGE and identified by Western blotting. Immunodecoration with anti-hInsR antiserum (A), anti-P-Tyr (B), anti-peptide antibodies directed against specific marker proteins, Rap1p (nuclei, C), Cdc35p (plasma membranes and cytoplasm, D), or Cdc24p (plasma membranes, E).

The finding that hInsRMy was autophosphorylated and able to phosphorylate yeast proteins on Tyr residues, whereas hInsRAc was not, is most plausibly explained by the assumption that hInsRMy associated with the plasma membrane and hInsRAc was cytoplasmic. To test this issue, cells were subfractionated as described in Section 2 (mitochondria and nuclei further purified by gradient centrifugation) and the fractions analyzed by Western blotting (Fig. 3). Sera directed against compartment-specific marker proteins were used to prove absence of mutual contaminations. Purity of nuclei was challenged by a serum against the general transcription factor, Rap1p, in Fig. 3C; anti-adenylate cyclase (Cdc35p) was specific for plasma membranes and cytoplasm in Fig.

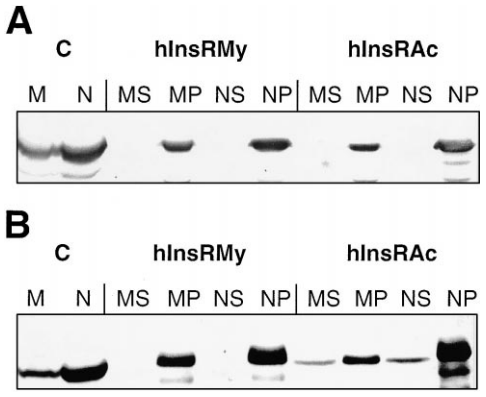

Fig. 4. Extraction of nuclei and plasma membranes of transformant cells of hInsRMy and hInsRAc with high salt (A) or alkali (B). Nuclei (N) or plasma membranes (M) (100 μg of protein each) were extracted with either 1 M NaCl or 0.2 M Na_2CO_3, pH 10.5 [27], and soluble extracts (S) or pellet (P) proteins were electrophoresed and immunodecorated as above using an anti-hInsR antiserum. C indicates the non-extracted control fraction.

3D, and anti-Cdc24p detected a protein present in plasma membranes in Fig. 3E. The vast majority of hInsRMy was found in the fraction containing nuclei, a smaller portion in plasma membranes, whereas the soluble supernatant (and mitochondria, not shown) did not contain detectable amounts of the protein (Fig. 3A). Surprisingly, it turned out that hInsRAc was only partly cytoplasmic. The majority of this protein also occurred in nuclei and about equal amounts were found in the plasma membrane fraction and in the cytoplasm. Mitochondria (not shown) were definitely devoid of the protein. hInsRMy was autophosphorylated, when associated with either plasma membranes or nuclei. Also Tyr-phosphorylated yeast proteins could be found in these two fractions, whereas only a very few minor substrate proteins were detected by the anti-P-Tyr antiserum in the cytoplasmic fraction (Fig. 3B). hInsRAc failed to autophosphorylate or to phosphorylate other proteins.

To determine the nature of the membrane association of the two versions of the hInsR protein, plasma membranes and nuclei were extracted with high salt or alkali [27]. No material of hInsRMy and only a minor fraction of hInsRAc could be extracted under either condition (Fig. 4A,B). Accordingly, neither of the proteins was loosely attached to the respective membrane, but rather behaved as a membrane protein. This was unexpected for hInsRAc and suggested that it was either inserted into the membrane by a lipophilic stretch of amino acids or, since this was not apparent from the primary structure, more likely, by rather stable protein–protein interactions.

The above results demonstrate that the prerequisites for a functional coupling of the heterologously expressed hInsR to putative downstream glucose signalling components of yeast were fulfilled: hInsRMy was located at the plasma membrane and in the nucleus and was constitutively autophosphorylated and capable of phosphorylating endogenous target proteins on Tyr residues. We tested functional coupling in yeast and examined whether the expression of active hInsR had any physiological consequences by measuring growth rates, respiration and glycogen accumulation on various carbon sources. We did not detect any significant differences to the wild-type transformed with a control plasmid on either glucose or non-fermentable carbon sources. In addition, iodine vapor staining revealed no differences between wild-type and transformant indicating that deposition of glycogen was about the same in the two strains (data not shown). This suggested that the heterologous protein did not interfere with the intrinsic yeast system of glucose signalling. This finding was in line with the observation that the pattern of Tyr-phosphorylated target proteins was not influenced by the carbon source (see above).

4. Discussion

The soluble cytoplasmic protein tyrosine kinase domain of hInsR has previously been expressed in the baculovirus expression system (Sf9 insect cells) in active form. These studies were undertaken in order to identify autophosphorylated tyrosine and serine/threonine residues [18,36] in InsR (in the absence of insulin) and analyze their importance in tyrosine kinase activity and signal transduction [14–17,37]. Here, we describe the stable expression of the complete cytoplasmic protein fragment of hInsR including the juxtamembrane domain in yeast cells. The protein has an apparent molecular mass of 48 kDa. In addition, we show that this protein is capable of autophosphorylation. Cross-reactivity of the 48 kDa protein with the anti-InsR and either of the two anti-P-Tyr antisera as well as the rapid removal of these signals by YOP protein phosphotyrosine phosphatase proves that hInsRMy is autophosphorylated on tyrosine residues after heterologous expression in yeast cells. Furthermore, several endogenous yeast proteins are phosphorylated on tyrosine residues in a hInsR-dependent way. The degree of autophosphorylation correlates well with the efficiency of tyrosine phosphorylation of these intrinsic substrates corroborating that the protein tyrosine kinase activity of hInsR is stimulated by autophosphorylation as in the insect system.

The protein tyrosine kinase domain was N-terminally fused to either an *N*-acetylation signal as a protein stabilizing sequence or an MGC box to allow membrane attachment through N-terminal lipophilic modification by myristate and, presumably, palmitate as has been verified in yeast in the case of Gpa1p, the Gα subunit of the trimeric G protein in the pheromone response pathway [33]. N-terminal membrane anchorage of the hInsRMy construct was verified by cellular subfractionation and shown to greatly enhance autophosphorylation in vivo. Most plausibly, this is due to the reduction of degrees of freedom and velocity of protein diffusion leading to an increased probability of bimolecular interaction to allow autophosphorylation. In contrast, hInsRAc is not autophosphorylated, although a large fraction of hInsRAc also co-purified with plasma membranes and was not extracted by high salt or alkali. This behavior cannot be explained at present.

The occurrence of hInsR in yeast nuclei was unexpected. Interestingly, a fraction of ligand-bound insulin receptor has been reported to co-fractionate with nuclei from mammalian insulin-responsive cells [38]. Furthermore, microinjection of human insulin into nuclei of *Xenopus* oocytes has been demonstrated to elicit mitogenic effects, such as stimulation of DNA synthesis [39]. These observations imply a functional role of insulin receptors in the nucleus possibly exerted after receptor activation in response to insulin binding and endocytotic trafficking. Conversely, these observations from vertebrates imply that the recombinant cytoplasmic domain of the receptor is committed and sufficient for nuclear import in

yeast despite the absence of a transmembrane domain and the uncoupling from an extracellular signal.

Surprisingly, tyrosine phosphorylation of intrinsic proteins had no apparent impact on the physiology of the yeast cells measured as growth rate on glucose or non-fermentable carbon sources or on respiratory rates or glycogen accumulation. This suggested that the constitutive tyrosine kinase activity of the hInsR did not interfere with glucose signalling in yeast. The failure to detect significant physiological consequences of the expression of the constitutively active tyrosine kinase domain of hInsR in yeast could be due to (i) too low kinase activity, (ii) rapid dephosphorylation and thereby inactivation of the kinase or (iii) absence of a homologue of the major substrate proteins, IRS-1–4, from yeast, which mediates most responses to insulin in mammals [40]. The assay of intrinsic Tyr-phosphatases argues that Tyr-phosphorylated proteins are only gradually dephosphorylated in a yeast cell homogenate. It must be deduced that the yeast target proteins of hInsR play no role in glucose signalling in yeast. This makes explanation iii the most likely and argues that the kinase activity of hInsR fails to activate the major substrate polypeptide(s) of the putative yeast InsR. Such a protein domain fused to the receptor has been detected in the nematode *Caenorhabditis elegans*, which has been shown to harbor a complete insulin-like signalling cascade ([41], reviewed in [42]). Likely, in lower eukaryotes such as *C. elegans* and *Saccharomyces cerevisiae* the endogenous InsR/insulin receptor substrate-like molecules directly feed into the downstream developmental or glucose signalling pathways, respectively. In yeast, the downstream components can be activated by exogenous mammalian insulin [8,42] but apparently fail to interact with the hInsR. Possibly, co-expression of the kinase domain of hInsR and IRS-1 is required for functional coupling of the former to the glucose signalling cascade in yeast.

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