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Reconstitution of an Insulin Signaling Pathway in *Xenopus laevis* Oocytes: Coexpression of a Mammalian Insulin Receptor and Three Different Mammalian Hexose Transporters

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We report the functional expression of the mammalian muscle-adipocyte insulin-sensitive hexose transporter in *Xenopus laevis* oocytes. Oocytes microinjected with RNA synthesized in vitro showed enhanced hexose transport activity compared with uninjected controls. However, like the endogenous oocyte hexose transporter, activity was stimulated only twofold by 1 μM insulin. *X. laevis* oocytes injected with in vitro-synthesized RNA encoding the human insulin proreceptor expressed a functionally active insulin receptor that enhanced the insulin sensitivity of injected oocytes. This increase was not observed in oocytes expressing a mutant insulin receptor that lacked protein tyrosine kinase activity. In the presence of the coexpressed human insulin receptor, insulin induced a two- to threefold increase in hexose transport. The muscle-, brain-, and liver-type hexose carriers normally expressed in tissues with different responses to insulin exhibited the same insulin sensitivity when expressed in oocytes. This was observed whether or not the insulin signal was transduced through a coexpressed human insulin receptor or the endogenous oocyte insulin-like growth factor I receptor. We conclude that the expressed human insulin receptor is able to couple efficiently with preexisting postreceptor regulatory pathways in oocytes and that the regulation of hexose transport in these cells can be mediated through the combined actions of the expressed human insulin receptor and the endogenous oocyte insulin-like growth factor I receptor.

Transport of glucose in most mammalian cell types occurs by carrier-mediated facilitated diffusion down a concentration gradient (41, 48). Recent studies indicate that this facilitated hexose uptake is mediated by a family of structurally related integral membrane proteins. Thus, a number of putative hexose transport proteins that have marked differences in their patterns of tissue-specific expression have been cloned. These include a cDNA clone encoding a protein expressed largely, but not exclusively, in HepG2 hepatoma cells and the brain (3, 29); one expressed in the liver, the pancreatic islets, and the kidneys (13, 44); one expressed in fetal skeletal muscle (24); and another expressed almost exclusively in fat cells and in adult skeletal and cardiac muscles (2, 5, 12, 18, 21). Functional studies indicate that at least three of these proteins (HepG2 brain, liver, and adult skeletal muscle) are, indeed, hexose carriers (2, 37, 44, 45).

Comparison of the deduced amino acid sequences of the cloned transporters indicates homology in primary structure (2, 3, 5, 12, 13, 18, 21, 24, 29, 44). In addition, the hydropathy plots of the different transporters are virtually superimposable and compatible with the proposed model for the orientation of the HepG2 glucose transporter in the cell membrane (29, 44). Sequence similarity is higher in the putative hydrophobic membrane-spanning segments than in the extramembranous more hydrophilic domains. Thus, each transporter differs in the length and/or the amino acid sequence of the amino- and carboxyl-terminal domains, the extracellular loop (containing a putative glycosylation site) between proposed transmembrane domains 1 and 2, and the large intracellular loop between transmembrane domains 6 and 7 (12, 29).

One mechanism by which insulin may stimulate hexose uptake in tissues (fat and muscle) that are uniquely sensitive to insulin-promoted hexose transport is through the rapid and reversible translocation of hexose transporters from intracellular membranes to the plasma membrane (4, 23, 32, 41, 43). We have reported that translocation also occurs during insulin-stimulated transport in oocytes (45), although to a lesser extent. Recent evidence suggests that a hexose transport protein that is expressed primarily in fat and in adult skeletal muscle is the principal insulin-responsive transport protein, on the basis of its tissue localization and its complete translocation from an intracellular membrane pool to the adipocyte cell surface in response to insulin (2, 19). However, additional evidence suggests that the insulin sensitivity of a given glucose transporter is a complex function that depends on the transporter itself, as well as the cell in which the transport occurs. Thus, transfection studies indicate that the HepG2 brain glucose transporter is translocated to the cell membrane (from an intracellular pool) in an insulin-dependent manner when expressed in insulin-sensitive Chinese hamster ovary cells or 3T3-L1 adipocytes (1, 15). In addition, studies from our laboratory indicated that both the brain and liver hexose transporters are responsive to insulin when expressed in *Xenopus laevis* oocytes in a fashion similar to the endogenous oocyte hexose transporter (45).

We report herein the functional expression of the muscle-adipocyte mammalian hexose transporter in *X. laevis* oocytes and present additional evidence supporting the notion of cell-dependent regulation of insulin stimulation of hexose transport. Since the effect of insulin in oocytes is probably mediated through insulin-like growth factor I (IGF-I) receptors (20, 26, 35, 36, 45), we studied the insulin sensitivity of hexose uptake in cells coexpressing the human insulin receptor and each of three (brain, liver, and muscle) mammalian hexose transporters.

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MATERIALS AND METHODS

Plasmids. The cDNAs encoding the rat brain and liver hexose transporter have been described (3, 44, 45). The latter was generously provided by H. Lodish. Full-length skeletal muscle hexose transporter cDNA (2) was kindly provided by M. Birnbaum. Plasmids containing wild-type and mutant (Ala substituted for Lys-1018) human insulin receptor cDNAs have been described previously (6).

In vitro transcription. Plasmids containing the appropriate cDNA inserts were linearized with *Sal*I (brain transporter), *Mlu*I (liver transporter), *Xba*I (muscle transporter), or *Spe*I (human insulin receptor). The DNAs were recovered by phenol extraction followed by ethanol precipitation and were used for in vitro transcription of full-length capped transcripts (30, 45) with T7 (brain and liver transporter cDNAs) or T3 (muscle transporter and human insulin receptor cDNAs) RNA polymerases. The plasmid containing a cDNA insert encoding the muscle hexose transporter was also transcribed by using T7 RNA polymerase in order to obtain a full-length antisense RNA.

RNA injection and hexose uptake assays. *X. laevis* oocytes were obtained from mature frogs by treatment with collagenase, injected with RNA, and incubated for 1 to 4 days at 18°C in modified Barth medium (7, 9, 45). Hexose uptake was measured essentially as described in reference 45 by using 2-deoxy-D-[1,2-^{3}H] glucose (Amersham Corp.) and 0.5 to 1.0 mM 2-deoxy-D-glucose or 3-*O*-methyl-D-[1-^{3}H]glucose (Amersham) and 0.5 mM 3-*O*-methyl-D-glucose. In studies of the effect of insulin or IGF-I on hexose uptake, oocytes were incubated for 60 min in medium containing the appropriate concentrations of each ligand prior to measuring hexose uptake. For further details, see reference 45. The effects of D- and L-glucose and cytochalasin B on hexose uptake were assessed as previously described (45).

Immunoprecipitation. For the preparation of antisera, a unique synthetic peptide corresponding to amino acids 490 to 509 of the deduced sequence of the muscle glucose transporter (2) was synthesized on an automated peptide synthesizer (Applied Biosystems, Inc.). The peptide was coupled to Affi-gel 10 (Bio-Rad Laboratories), emulsified with complete Freund adjuvant, and injected into rabbits; this procedure was followed by two boosts in incomplete adjuvant. The antiserum immunoprecipitated a 40-kilodalton protein synthesized in vitro from synthetic mRNA encoding the muscle hexose transporter in a rabbit reticulocyte system. The [^{35}S]methionine-labeled 40-kilodalton protein was not observed when immunoprecipitation reactions were carried out in the presence of an excess (200 μg) of the peptide to which the antibody was elicited.

Oocytes were metabolically labeled in Barth solution containing 5 mCi of [^{35}S]methionine per ml (1,000 Ci/mmol; Amersham) for 24 h. Label was added 6 h after microinjecting the RNA. Oocytes were solubilized, immunoprecipitated by using the anti-peptide serum described above, and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography (45).

^{125}I-labeled insulin binding. Oocytes were incubated at 4°C in Barth medium containing 10 mg of bovine serum albumin per ml; 50 μg each of aprotinin, leupeptin, and soybean trypsin inhibitor per ml; ^{125}I-labeled insulin (5×10^{-11} M); and increasing concentrations of unlabeled insulin for 6 h. After washing in the same medium containing no insulin, oocytes were directly counted in a gamma counter to determine the amount of bound insulin. Nonspecific binding was determined in samples incubated in the presence of 200 nM unlabeled insulin and 20 nM IGF-I.

Autophosphorylation of the insulin receptor. Oocytes injected with synthetic RNA encoding the wild-type or the mutant human insulin receptors were homogenized in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfhonic acid (HEPES; pH 7.6) containing 2% Triton X-100; 0.15 M NaCl; 5 mM EDTA; 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA); 10 μg each of aprotinin, leupeptin, and soybean trypsin inhibitor per ml; and 0.2 mM phenylmethylsulfonyl fluoride. After centrifugation at 20,000 × *g* for 20 min, the supernatant fluid was immunoprecipitated with a 1/20 volume of anti-peptide serum AbP5 (16) at 4°C for 14 h. This antibody recognizes the carboxyl terminus of the β subunit of the human insulin receptor without inhibiting its intrinsic kinase activity (16). The immunocomplexes were adsorbed to protein A-Sepharose, washed in the above immunoprecipitation buffer, and suspended in 50 mM HEPES (pH 7.6) containing 0.1% Triton X-100; 0.15 mM NaCl; 10 μg each of aprotinin, leupeptin, and soybean trypsin inhibitor per ml; and 200 nM insulin. After 90 min at 4°C, the solution was adjusted to 4 mM Mn^{2+}, 12 mM Mg^{2+}, 2 mM dithiothreitol, 50 μM ATP, and 50 μCi of [γ-^{32}P]ATP and incubated for 15 min at 20°C. The immune complexes were collected by centrifugation, washed, and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radioautography (16, 40).

Oocyte maturation. Groups of 50 oocytes each were incubated in modified Barth medium lacking potassium ions and containing 0.5 mg of bovine serum albumin per ml, in the absence or presence of insulin. After 20 h at 18°C, oocytes were scored by the appearance of a white spot in the animal pole. Oocytes were also dissected under the microscope and examined for the presence or absence of the germinal vesicle (26).

RESULTS

Expression of the mammalian adult muscle hexose transporter in *X. laevis* oocytes and the effect of insulin on hexose uptake. Figure 1 shows an immunoprecipitation analysis of total homogenates prepared from *X. laevis* oocytes that had been injected with RNA encoding the muscle transporter. The antibodies were raised against a unique synthetic peptide corresponding to the deduced sequence of the COOH-terminal 20 amino acid residues of this protein (Leu-490 to Asp-509). The antiserum immunoprecipitates a protein that migrates with an apparent M_r of 40,000 to 45,000 (Fig. 1, lane 1) that is not present in uninjected oocytes (Fig. 1, lane 5). The specificity of the antiserum (see also Materials and Methods) is indicated by the observation that this labeled material was not immunoprecipitated in the presence of an excess of the peptide Leu-490 to Asp-509 (Fig. 1, lanes 2 and 3) or by preimmune serum (Fig. 1, lane 4).

To determine the time course of expression of the muscle transporter in injected oocytes, we measured uptake of 2-deoxy-D-glucose at different times after the injection of the RNA. As shown in Fig. 2, the uptake of 2-deoxy-D-glucose was markedly enhanced in oocytes injected with RNA encoding the transporter, as compared with oocytes injected with water. This increase in transport was observed as early as 24 h after injection and increased linearly for up to 4 days (see Fig. 2). When 2-deoxy-D-glucose uptake was measured 4 days after injection, microinjected oocytes transported 2-deoxy-D-glucose at a velocity 15-fold greater than that of controls injected with water. There was no detectable in-

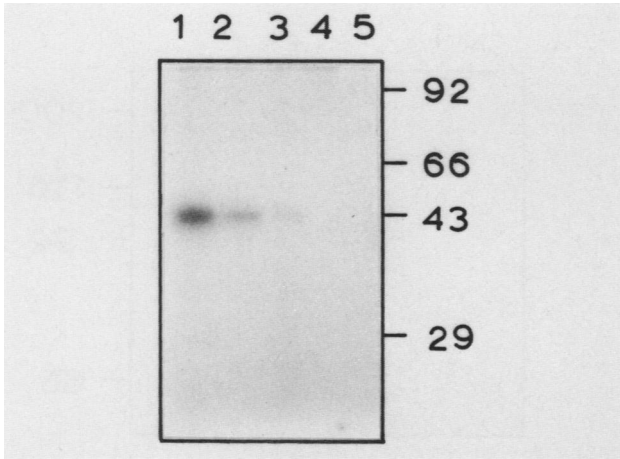

FIG. 1. Expression of the rat skeletal muscle glucose transporter in *X. laevis* oocytes. Oocytes were injected with 40 ng of synthesized RNA encoding the rat skeletal muscle glucose transporter and were incubated for 24 h in Barth medium in the presence of [^{35}S]methionine. Oocytes were solubilized and immunoprecipitated with preimmune serum (lane 4) or an anti-peptide serum elicited in rabbits against a peptide (Leu-490 to Asp-509) containing the last 20 carboxyl-terminal amino acids of the deduced sequence of the rat skeletal muscle glucose transporter (lanes 1, 2, 3, and 5). Lanes 1 to 4, oocytes injected with the RNA; lane 5, uninjected oocytes. The immunoprecipitation reactions were carried out in the absence (lanes 1, 4, and 5) or in the presence of 10 (lane 2) or 100 (lane 3) μg of the peptide Leu-490 to Asp-509. Positions of molecular weight markers (10^3) are shown on the right.

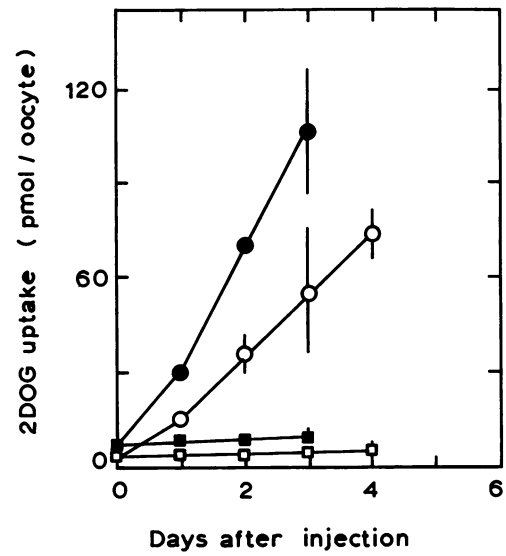

FIG. 2. Functional expression of the rat skeletal muscle hexose transporter in *Xenopus* oocytes. Oocytes were injected with 10 ng of RNA encoding the rat skeletal muscle hexose transporter per oocyte (●,○), and 2-deoxy-D-glucose (2DOG) uptake was determined at different times thereafter. Controls (■,□) were injected with water. Before measuring uptake, oocytes were incubated for 60 min at 18°C in the presence (●,■) or in the absence (○,□) of 1 μM insulin, as indicated in Materials and Methods. Data represent the mean and standard error of the mean of three to four groups of 30 oocytes each.

crease in uptake when oocytes were injected with an antisense RNA transcribed in vitro from the cDNA encoding the muscle hexose transporter. The values of 2-deoxy-D-glucose uptake in 10-min assays were 7.5 and 6.8 pmol per oocyte for oocytes injected 3 days before with antisense RNA (10 ng per oocyte) and water, respectively.

As expected, uptake of 2-deoxy-D-glucose in oocytes injected with RNA encoding the muscle transporter was sensitive to inhibition by cytochalasin B, a strong and specific inhibitor of facilitated hexose uptake in mammalian cells (41, 48), with an apparent K_i of 0.6 μM. In addition, competition studies using L- and D-glucose indicated that the enhanced 2-deoxy-D-glucose activity detected in oocytes injected with RNA is stereospecific. In uptake assays carried out 2 days after injection, 2-deoxy-D-glucose uptake activity was 36.5 pmol per oocyte in 10 min. No significant change in the level of transport was observed when the uptake assay was carried out in the presence of 0.5 M L-glucose (32.3 pmol per oocyte). On the other hand, the uptake observed when the transport assay was carried out in the presence of 0.5 M D-glucose was 5.1 pmol per oocyte. Taken together, these results demonstrate that the mammalian muscle hexose transporter can be expressed in *X. laevis* oocytes in a functionally active state.

Since hexose uptake in *Xenopus* oocytes is sensitive to high concentrations of insulin (20, 45) and both the rat brain and liver hexose transporters, which are not insulin sensitive in the tissues from which they were cloned, respond to insulin when expressed in *X. laevis* (45), we decided to test whether insulin affected the activity of the muscle hexose transporter expressed in oocytes. Recent reports indicate that this protein, which is expressed primarily in adult muscle and adipocytes, exhibits the properties expected for the insulin-sensitive hexose transporter (2, 18, 19).

Insulin stimulated the uptake of 2-deoxy-D-glucose in oocytes expressing the muscle transporter (Fig. 2). However, the response to insulin was similar to that observed for the endogenous oocyte transporter, e.g., exposure of oocytes to 1 μM insulin increased the rate of transport approximately twofold (Fig. 2). A twofold activation in transport was also observed when uptake of 3-*O*-methylglucose was measured in injected and uninjected oocytes (data not shown). This is in striking contrast to the 20- to 30-fold increase in the velocity of hexose transport observed in adipocytes after insulin treatment (23, 41). Interestingly, hexose transport in oocytes injected with either the rat brain or liver hexose transporter is also stimulated approximately twofold by insulin (45).

Functional effects of the expression of the human placental insulin receptor in *X. laevis* oocytes. The results presented above indicate that mammalian hexose transporters, when expressed in oocytes, respond to insulin to about the same degree, qualitatively, as the endogenous oocyte transporter (see reference 45). We consistently observed a twofold increase in hexose transport after incubating the oocytes in the presence of 1 μM insulin. However, we did not see any increase in hexose transport when the oocytes were treated with 10 nM insulin, a concentration of insulin that normally elicits a full response in insulin-sensitive mammalian tissues (23, 41). Since there is evidence that *Xenopus* oocytes have few if any functional insulin receptors on their cell surface but instead possess IGF-I receptors (20, 26, 35, 36, 45), it seemed reasonable to surmise that the high concentrations of insulin needed to activate hexose transport in oocytes might be due to activation of oocyte IGF-I receptors. To address this, we decided to find out whether the expression of functional human insulin receptors in *Xenopus* oocytes would render these cells sensitive to lower concentrations of

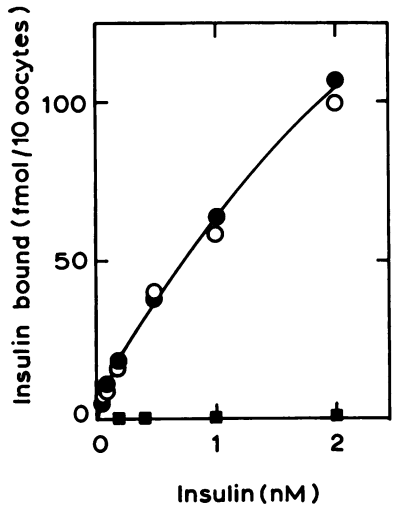

FIG. 3. Insulin binding to *X. laevis* oocytes. Oocytes were injected with 10 ng of RNA encoding the wild type (●) or a mutant (Lys-1018 replaced by Ala) (○) insulin receptor and were incubated for 48 h in Barth medium. Controls (■) were injected with water. Injected cells were exposed to 0.05 nM ^{125}I-labeled insulin, with different concentrations of unlabeled insulin, for 8 h at 4°C, washed, and counted as described in Materials and Methods.

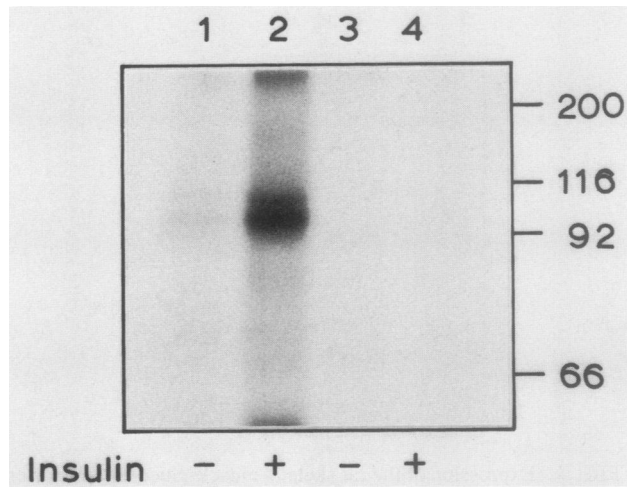

FIG. 4. Insulin-stimulated autophosphorylation of human insulin receptors expressed in *Xenopus* oocytes. Homogenates of oocytes expressing the wild-type (10 fmol of insulin-binding activity, lanes 1 and 2) or the mutant (20 fmol of insulin-binding activity, lanes 3 and 4) human placental insulin receptor were immunoprecipitated with anti-peptide antibody AbP5 and subjected to autophosphorylation (see Materials and Methods). Assays were performed in the presence (+) or in the absence (−) of 100 nM insulin. The major band in lane 2 has an M_r of about 95,000. Positions of molecular weight markers (10^3) are shown on the right.

insulin and what the effect might be on the regulation of hexose transport.

Oocytes were injected with in vitro synthesized RNA encoding the human placental insulin receptor. To test whether this RNA is translated in *Xenopus* oocytes, injected and uninjected cells were assayed for insulin binding. Preliminary experiments indicated that the time required for maximal insulin binding was 6 h at 4°C. Figure 3 provides data obtained by measuring ^{125}I-labeled insulin binding to injected and uninjected oocytes. Very low levels of specific binding of insulin were detected in uninjected oocytes. By contrast, oocytes injected with 10 ng of RNA encoding the human insulin receptor showed a dramatic increase in the binding of ^{125}I-labeled insulin (Fig. 3). The number of insulin-binding sites per cell was similar in oocytes injected with RNA encoding either the wild-type human insulin receptor or a human receptor mutated in the ATP-binding site (Fig. 3). These results were obtained two days after RNA injection. There was no further increase in the number of insulin-binding sites after 3 or 4 days of culture (data not shown).

To test whether the oocytes expressed the human insulin receptor in a functionally active state, we evaluated the protein kinase activity of the human receptor by studying insulin-dependent autophosphorylation after immunoprecipitation with human insulin receptor-specific anti-peptide antibody AbP5 (16). As expected, insulin stimulated the phosphorylation of the β subunit of the wild-type human receptor expressed in *X. laevis* (Fig. 4, lanes 1 and 2), but the mutant human receptor did not autophosphorylate (Fig. 4, lanes 3 and 4).

We next studied the induction of cell division by insulin, scoring germinal vesicle breakdown of oocytes exposed to insulin for 20 h. Under these conditions, the maturation responses of uninjected oocytes to 10, 100, and 1,000 nM insulin were 0, 30, and 90%, respectively. On the other hand, oocytes expressing the wild-type human insulin receptor were clearly more sensitive to insulin, with 30 and 80% maturation induced by exposing the oocytes to 1.0 and 10 nM insulin, respectively. No increase in the sensitivity to insulin was observed in oocytes injected with 10 ng of RNA encoding the mutant human insulin receptor (data not shown).

Uptake of 2-deoxy-D-glucose in response to treatment with either insulin or IGF-I was measured in uninjected and injected oocytes (Fig. 5). As described previously (20, 45), uninjected oocytes that are not expressing the human insulin receptor respond to 1 μM insulin with a 1.7- to 2.0-fold increase in hexose transport, with half-maximal stimulation occurring at 100 to 200 nM insulin (Fig. 5A). When IGF-I was used, a similar increase was elicited by 1 to 2 nM IGF-I, with a half-maximal response at 0.2 nM (data not shown and reference 45). Interestingly, the response of oocytes expressing the wild-type insulin receptor to insulin was substantially different from that observed in uninjected oocytes (Fig. 5A). A two- and threefold increase in transport was observed in the presence of 10 nM and 1 μM insulin, respectively, and half-maximal stimulation occurred at about 5 nM insulin (Fig. 5A). This was accompanied by a significant increase in the basal transport activity in the absence of insulin, 22 pmol per oocyte for injected cells in a 10-min standard assay, compared with 7 pmol per oocyte in uninjected cells (Fig. 5A). On the other hand, sensitivity to IGF-I was similar in injected and uninjected oocytes, with half-maximal activation occurring at about 0.2 nM IGF-I (Fig. 5B; reference 45). As expected, the basal level of transport in the absence of IGF-I in this experiment was also increased in injected cells (18 pmol per oocyte) as compared with uninjected controls (7 pmol per oocyte) (Fig. 5B).

The above results add credence to the suggestion of heterologous activation of the oocyte IGF-I receptor by high concentrations of insulin. To further study this, we measured 2-deoxy-D-glucose uptake in oocytes expressing the human insulin receptor but preincubated in the presence of 2 nM insulin together with 2 nM IGF-I. There was a 2.5-fold increase in hexose uptake under these conditions (50 pmol

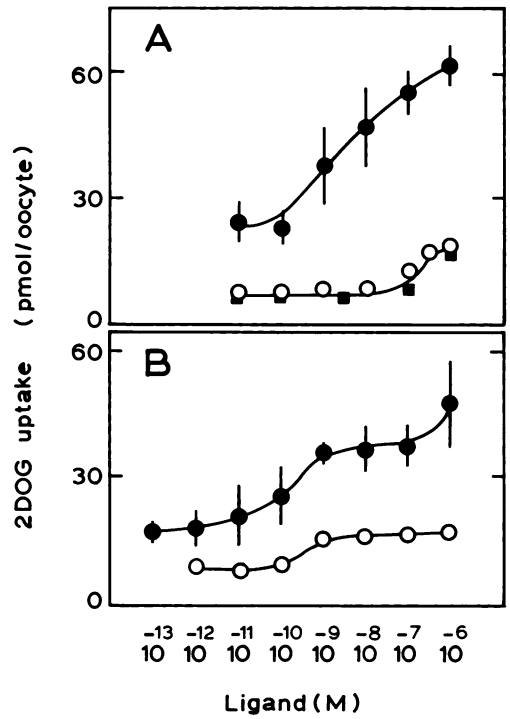

FIG. 5. Stimulation of 2-deoxy-D-glucose uptake by insulin or IGF-I in *Xenopus* oocytes expressing human placental insulin receptors. Oocytes were incubated in Barth medium containing 0.05% bovine serum albumin and the indicated concentrations of insulin (A) or IGF-I (B). After incubation for 60 min at 18°C, 2-deoxy-D-glucose (2DOG) uptake was assayed. Each point represents the mean and standard error of the mean of three to four groups of 30 oocytes each. Symbols: ●, oocytes injected with RNA encoding the wild-type human placental insulin receptor; ○, oocytes injected with RNA encoding the mutant placental insulin receptor; ■, uninjected oocytes.

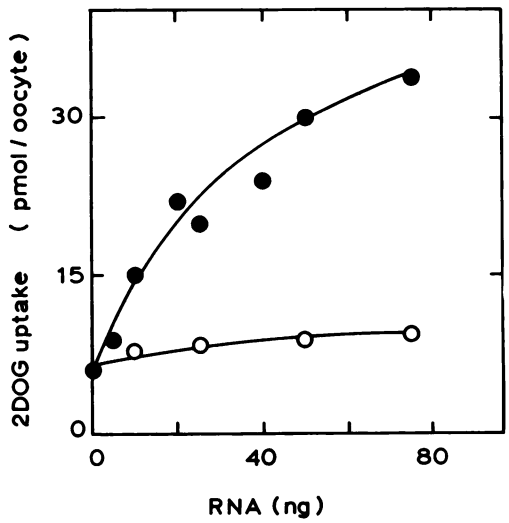

FIG. 6. Basal hexose uptake activity in oocytes injected with different amounts of RNA encoding the wild-type (●) or the mutant (○) human insulin receptor. The indicated amounts of RNA were injected per oocyte, and, after a 2-day incubation period, 2-deoxy-D-glucose (2DOG) uptake was measured. Each point represents the average of two groups of 30 oocytes each.

per oocyte) as compared with cells incubated in the absence of both ligands (19 pmol per oocyte). Only a 1.7-fold increase in 2-deoxy-D-glucose uptake was observed when this experiment was repeated with uninjected oocytes (6 and 10.3 pmol per oocyte for cells incubated in the absence or presence of 2 nM insulin plus 2 nM IGF-I). An equivalent result (1.7-fold activation) was obtained when uninjected cells were stimulated with 2 nM IGF-I alone, and activation was not observed in cells treated with just 2 nM insulin (see also reference 45).

No changes in the effects of either insulin or IGF-I on hexose transport were observed in oocytes injected with RNA encoding the mutant kinase-defective human insulin receptor. This was the case for transport in the absence of insulin as well as for full activation in the presence of 1 μM insulin or 10 nM IGF-I (Fig. 5A and B). The effects of injecting increasing amounts of RNA encoding either the wild-type or mutant insulin receptor into oocytes are depicted in Fig. 6. In the former case, increasing the amount of RNA per oocyte produced a clear, although nonlinear increase in the basal level of hexose uptake in the absence of a stimulating ligand. ^{125}I-labeled insulin-binding studies confirmed that the increased transport activity was accompanied by concomitant increases in the insulin-binding activities of the injected oocytes of 25, 40, and 100 fmol per oocyte for 10, 25, and 75 ng of RNA injected, respectively (data not shown). By contrast, there was no significant change in basal hexose transport activity in oocytes injected with RNA encoding the mutant human insulin receptor (Fig. 6), although the expected increase in ^{125}I-labeled insulin-binding was observed.

Effect of insulin on hexose uptake by oocytes coexpressing human placental insulin receptors and different mammalian hexose transporters. The above results demonstrate that oocytes expressing the human insulin receptor respond to physiological concentrations of insulin with an increase in hexose uptake. We then asked whether coexpression of the human insulin receptor and either the brain, liver, or muscle mammalian hexose transporters would allow us to detect any functional difference in insulin sensitivity among the three transporters.

As shown in Fig. 7, cells expressing any one of the three mammalian hexose transporters did not respond to 10 nM insulin, but all responded to 1 μM insulin with a 1.7- to 2.0-fold increase in transport (Fig. 7A). A similar response to insulin was observed in uninjected oocytes (Fig. 7A and 7B). On the other hand, cells coexpressing both the human placental insulin receptor and a mammalian transporter responded to 10 nM insulin with a twofold increase in hexose transport and to 1 μM insulin with a threefold increase in transport (Fig. 7B). Similar results were observed in oocytes injected only with RNA encoding the human insulin receptor (Fig. 7B). In addition, equivalent results were obtained, in terms of sensitivity to insulin and degree of activation of uptake, when transport of 3-*O*-methylglucose in oocytes coexpressing the human placental insulin receptor and the mammalian glucose transporters was measured (data not shown). As a control, hexose transport in oocytes expressing the muscle transporter and the mutant human insulin receptor was evaluated. There was no effect of 10 nM insulin on hexose transport, and the activation of transport at 1 μM insulin (about twofold) was comparable to that observed in oocytes injected with the muscle transporter alone.

We then reexamined the possibility of an additive effect of insulin on the activation of hexose uptake mediated by the expressed human insulin receptor and the endogenous

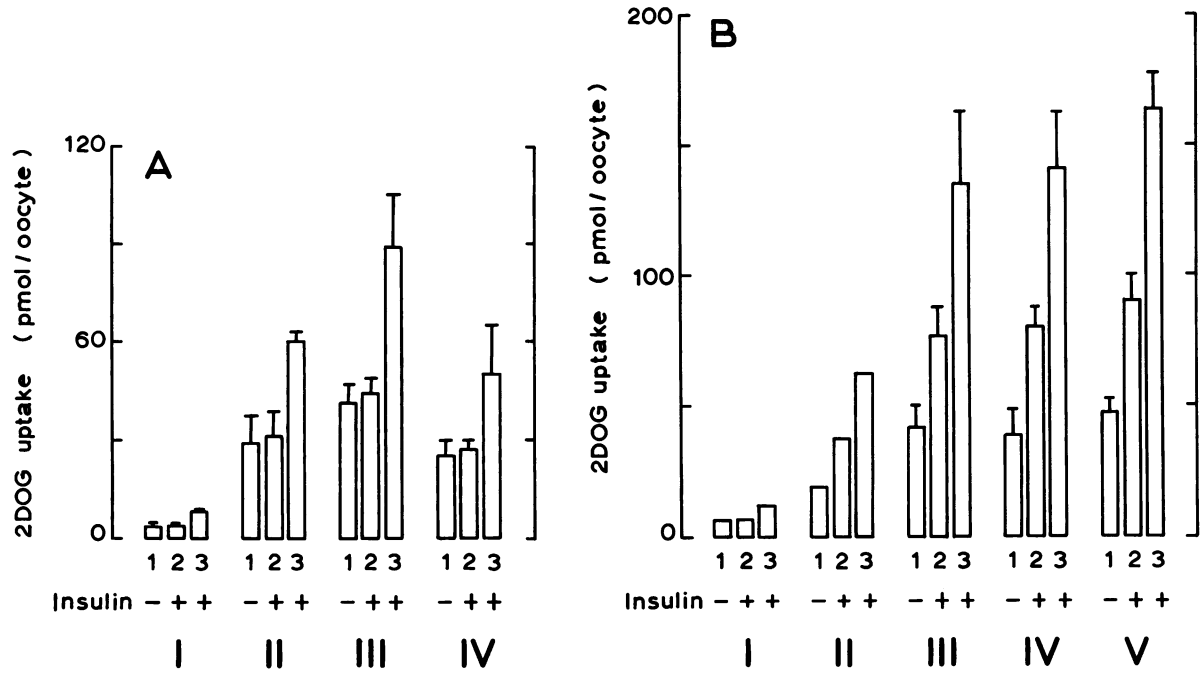

FIG. 7. Effect of insulin on hexose uptake by injected *Xenopus* oocytes. (A) Insulin effect on 2-deoxy-D-glucose (2DOG) uptake by oocytes injected with water (set I) or with 10, 20, or 10 ng of RNA encoding the rat brain (set II), liver (set III), or skeletal muscle (set IV) hexose transporter. Assays were performed 2 days after injecting the RNA. Prior to measuring hexose uptake, oocytes were incubated in the absence (bars 1) or the presence of 10 nM (bars 2) or 1 μM (bars 3) insulin. Data represent the mean and standard error of the mean of three groups of 20 to 30 oocytes each. (B) Insulin effect on 2-deoxy-D-glucose (2DOG) uptake by uninjected oocytes (set I); oocytes injected with 10 ng of RNA encoding the human insulin receptor (set II); or oocytes injected simultaneously with 10 ng of RNA encoding the human insulin receptor and 10 ng of RNA encoding the rat brain (set III), liver (set IV), or skeletal muscle (set V) glucose transporter. For other details, see panel A. Each set consists of three bars and is numbered (I to V) from the left.

oocyte IGF-I receptor. Oocytes expressing the muscle hexose transporter showed approximately a 1.7-fold increase in hexose transport after preincubation in the presence of 2 nM IGF-I (Fig. 8A). An equivalent result, 1.8-fold activation, was obtained when oocytes were stimulated with 1 μM insulin; however, there was no stimulation with 2 nM insulin (Fig. 8A). When this experiment was repeated with oocytes coexpressing the muscle transporter and the human insulin receptor, either 2 nM insulin or 2 nM IGF-I elicited a 1.7-fold increase in 2-deoxy-D-glucose uptake (Fig. 8B). Moreover, preincubation of the oocytes with 2 nM IGF-I plus 2 nM insulin resulted in 2.9-fold increase in transport. This result is consistent with the possibility of an additive or synergistic mechanism involving both the expressed human insulin receptor and the endogenous oocyte IGF-I receptor.

DISCUSSION

We have shown that *X. laevis* oocytes injected with RNA encoding a mammalian hexose transporter expressed principally in tissues whose hexose transport is activated by insulin (muscle and fat) synthesize a protein with the size, immunological characteristics, and properties expected for the muscle-adipocyte hexose transporter (2, 18, 41, 48). The temporal pattern of expression of this transport activity in injected oocytes, that is, the steady increase in hexose transport activity for at least 4 days, is similar to that described previously for the rat brain and liver glucose transporter in oocytes and probably reflects the stability of the microinjected RNAs (45). The 15-fold increase in the velocity of hexose uptake of oocytes microinjected with RNA encoding the muscle transporter, as compared with uninjected controls, is also similar to the increase observed in oocytes injected with equivalent amounts of RNA encoding either the rat brain or liver hexose transporter (45).

Oocytes injected with RNA encoding the human insulin receptor showed a significant increase in the number of insulin-binding sites on their cell membrane. This increase was detectable as early as 24 h after injection of the RNA and corresponded to an increase of 100- to >1,000-fold in the number of sites, the exact increase depending on the amount of RNA injected. Our data also showed that the human insulin receptor was expressed in a functionally active form in oocytes, as evidenced by its capacity to autophosphorylate in response to insulin and to carry out two insulin-mediated events: germinal vesicle breakdown and hexose transport. In contrast, oocytes injected with RNA encoding a mutant receptor without intrinsic kinase activity (6) showed an increase in insulin-binding sites but no transmembrane signaling.

Insulin stimulates hexose transport mediated by the expressed mammalian muscle transporter in oocytes. However, the extent of stimulation is in no way equivalent to the 20- to 30-fold increase in hexose transport observed after insulin treatment of adipocytes (23, 41). In fact, the extent (or fold) stimulation by insulin of the muscle transporter expressed in oocytes is similar to the stimulation of the endogenous oocyte transporter (20, 45). Moreover, both the rat brain and liver hexose transporters, which are primarily expressed in cells that are not sensitive to insulin-stimulated hexose transport, also respond to insulin with a twofold increase in hexose transport when expressed in oocytes (see this report and reference 45). In this regard, the possibility

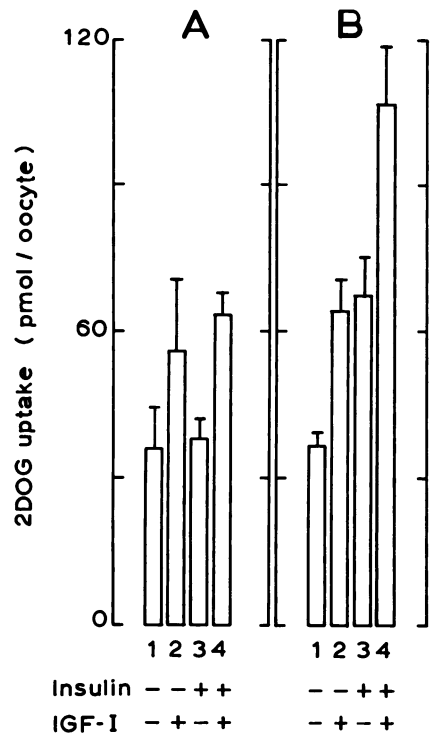

FIG. 8. Effect of low concentrations of insulin and IGF-I on 2-deoxy-D-glucose (2DOG) uptake by oocytes injected with 10 ng of RNA encoding the rat skeletal muscle hexose transporter (A) or by oocytes injected simultaneously with 10 ng of RNA encoding the human insulin receptor and 10 ng of RNA encoding the rat skeletal muscle hexose transporter (B). Assays were performed 2 days after injection. Prior to measuring hexose uptake, oocytes were incubated without any ligand (bars 1), in the presence of 2 nM of IGF-I (bars 2) or insulin (bars 3), or in the presence of 2 nM each IGF-I and insulin (lanes 4). Data represent the mean and standard error of the mean of 3 to 4 groups of 20 to 30 oocytes each.

exists that the activation of hexose uptake by insulin in *Xenopus* oocytes might be the expression of a very general effect of the hormone on all cell surface membrane proteins. However, the evidence available appears to indicate that insulin exerts a specific effect on certain membrane-bound enzymes. Thus, insulin stimulates a high-K_m phosphodiesterase activity present in *Xenopus* oocytes but has no effect on the respective low-K_m enzyme also present on the membranes of these cells (36). It is also interesting that insulin does not activate, but instead appears to inhibit, the activity of a membrane-bound adenylate cyclase in *Xenopus* oocytes (36). In addition, we have preliminary evidence that indicates that the activity of a member of the mammalian multidrug resistance proteins expressed into *Xenopus* oocytes is not affected by treatment of the oocytes with insulin (G. Castillo, J. C. Vera, and O. M. Rosen, unpublished results). These observations give credibility to the idea of a specific effect of insulin on hexose transport in oocytes and are consistent with the notion that the stimulation of a given transporter is dependent on the type of cell in which the protein is being expressed. Further support for this hypothesis is provided by reports that both the HepG2 and the brain hexose transporters respond to insulin when expressed in mammalian cells in which hexose uptake is regulated by insulin (1, 15). In addition, and in complete accordance with our own observations, these reports also indicate that the insulin sensitivity of introduced transporters is similar to that of the endogenous transporter present in the cells under study (1, 15).

Hexose transport in injected or uninjected oocytes was not sensitive to 1 to 10 nM insulin. High concentrations of insulin (1 μM) were needed to produce a maximal effect on transport (see also reference 45). This is probably a property inherent in the oocytes and not specifically related to hexose transport (20, 26, 35, 36, 45). The requirement for micromolar concentrations of insulin is consistent with the notion that the effects of insulin on *Xenopus* oocytes are mediated through IGF-I receptors and that the oocytes have very few high-affinity insulin receptors (20, 26, 35, 36, 45). Insulin and IGF-I receptors, like their ligands, are closely related molecules. In many cells, they mediate similar biological events and can be activated by the heterologous ligand. In this connection, our results indicate directly that *Xenopus* oocytes express a low concentration of insulin-binding sites on their surface. A previous report indicated the presence of a high content of insulin receptors on the surface of *X. laevis* oocytes, with a density on the membrane at least 10-fold higher than that which is found in liver cells (22, 26, 46). However, functional studies failed to support this observation and indicated lack of response of *X. laevis* oocytes to low concentrations of insulin (26, 35, 36).

Evidence obtained in mammalian cells expressing different levels of insulin receptors encoded by transfected cDNA has established a correlation between the insulin sensitivity of insulin-responsive cells, e.g., the minimal concentration of insulin required to induce a given response, and the number of insulin receptors expressed on their cell membranes (10, 11, 14, 17, 25, 38, 39, 42, 49, 50). Our results demonstrate that this is also true in oocytes expressing the human insulin receptor. Such oocytes are able to couple with preexisting postreceptor regulatory pathways, probably common to both IGF-I and insulin signaling mechanisms, as evidenced by an increased insulin sensitivity of the injected oocytes. Enhanced responsivity was observed when two well-characterized responses to insulin were studied: induction of maturation and hexose uptake (20, 26, 35, 36, 45). In both cases, at least 100-fold less insulin was needed to elicit equivalent responses in oocytes expressing the human insulin receptor compared with control oocytes. In addition, expression of the wild-type human insulin receptor (but not the mutant receptor) in oocytes produced a significant increase in the basal level of hexose uptake, in the absence of any ligand. The increase correlated with the number of active insulin receptors expressed on the cell surface. This observation has been also reported in mammalian cells expressing high levels of transfected insulin receptors (11, 17, 38, 39, 50). Although the explanation is not clear, it may be that the ligand-free receptor is slightly active or otherwise activable so that when present in high concentrations, partial insulin effects are observed.

An analysis of the insulin dose-response curve for activation of hexose uptake in oocytes expressing the human insulin receptor indicates that in addition to increased sensitivity to insulin, the transport activity becomes somewhat more responsive to insulin, with a threefold activation in transport observed at 1 μM insulin. Based on the insulin dose-response curve for the activation of hexose uptake in uninjected oocytes, we suggest the possibility that at high concentrations of insulin, the ligand is exercising its effect by binding to the human insulin receptor and to the endogenous oocyte IGF-I receptor (8, 20, 26, 33, 35, 36, 42, 45). Thus, the full response of *X. laevis* to insulin, at least in terms of

hexose uptake, may require occupation of both kinds of receptors by ligand. Similar results have been reported in mammalian cells (17, 33). Support for this hypothesis is provided by the 2.5- to 3.0-fold activation of hexose uptake observed in the presence of both ligands at concentrations that preclude heterologous binding.

The functional effects discussed above were not observed in oocytes expressing equivalent levels of mutated receptors (with Lys-1018 replaced by Ala; see reference 6). This mutant receptor, when expressed in mammalian cells, lacks tyrosine kinase activity and fails to mediate several biological responses to insulin, including insulin-dependent autophosphorylation, activation of hexose uptake, S6 kinase activation, endogenous substrate phosphorylation, thymidine incorporation into DNA, and down regulation of the receptor (6, 28, 34). Not unexpectedly, the mutant receptor expressed in oocytes does not autophosphorylate in response to insulin and does not mediate insulin-stimulated hexose uptake and oocyte maturation, suggesting that in these cells the tyrosine kinase activity of the receptor, or its autophosphorylated state, is necessary for these physiological responses to insulin. However, and as reported previously (34), the wild-type and the mutated receptor bind insulin equivalently.

In conclusion, our results indicate that the mammalian brain, liver, and adult skeletal muscle-adipocyte hexose transporters exhibit the same degree of activation by insulin when coexpressed with the human insulin receptor in *X. laevis* oocytes. The two differences between oocytes expressing and not expressing the human insulin receptor are a marked diminution in the concentration of insulin needed for activation and a boost (from twofold to threefold) in the maximum stimulation of transport by either high concentrations of insulin or low concentrations of insulin plus IGF-I. In oocytes coexpressing the human insulin receptor and each of the three transporters, 10 nM insulin induces a twofold increase in hexose transport and 1 μM insulin further increases it to about threefold. Thus, the three different mammalian hexose carriers are functionally very similar with respect to their sensitivities to insulin when expressed in oocytes, whether or not the insulin signal is transduced through a coexpressed human insulin receptor or the endogenous oocyte IGF-I receptor. In addition, their insulin sensitivities are similar to that of the endogenous oocyte hexose carrier (see also reference 45). This is in striking contrast to the differences observed in the degree to which insulin affects hexose transport in tissues expressing each of these transporters (2, 19, 26, 41). These, and previous observations (1, 15), suggest that the insulin responsiveness of a given hexose transporter is dependent on the cellular context in which the protein is being expressed and may be dependent on the participation of additional, as yet undefined, tissue- or cell-specific molecules involved in the regulation of hormone-induced changes in transport. This hypothesis also implies that the differences in primary structure of the mammalian hexose transporters (12, 41) may be important in defining this regulatory pathway. This is particularly relevant to those tissues or cells, such as adipocytes, which express two or more hexose transporters that respond differently to insulin (15, 18, 19, 31, 47, 51).

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