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In situ autoradiography and ligand-dependent tyrosine kinase activity reveal insulin receptors and insulin-like growth factor I receptors in prepancreatic chicken embryos

(development/signal transduction)

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ABSTRACT **We previously reported specific cross-linking of ^{125}I-labeled insulin and ^{125}I-labeled insulin-like growth factor I (IGF-I) to the α subunit of their respective receptors in chicken embryos of 20 somites and older. To achieve adequate sensitivity and localize spatially the receptors in younger embryos, we adapted an autoradiographic technique using whole-mounted chicken blastoderms. Insulin receptors and IGF-I receptors were expressed and could be localized as early as gastrulation, before the first somite is formed. Relative density was analyzed by a computer-assisted image system, revealing overall slightly higher binding of IGF-I than of insulin. Structures rich in both types of receptors were predominantly of ectodermal origin: Hensen's node in gastrulating embryos and neural folds, neural tube and optic vesicles during neurulation. The signal transduction capability of the receptors in early organogenesis was assessed by their ability to phosphorylate the exogenous substrate poly(Glu^{80}Tyr^{20}). Ligand-dependent tyrosine phosphorylation was demonstrable with both insulin and IGF-I in glycoprotein-enriched preparations from embryos at days 2 through 6 of embryogenesis. There was a developmentally regulated change in ligand-dependent tyrosine kinase activity, with a sharp increase from day 2 to day 4, in contrast with a small increase in the ligand binding. Binding of ^{125}I-labeled IGF-I was, with the solubilized receptors, severalfold higher than binding of ^{125}I-labeled insulin. However, the insulin-dependent phosphorylation was as high as the IGF-I-dependent phosphorylation at each developmental stage.**

Insulin and insulin-like growth factor I (IGF-I) are members of a family of peptides which regulate metabolic pathways, mitogenesis, and cell differentiation through (at least) two receptor types, the insulin receptor and the IGF-I receptor (1–3). The two receptors possess intrinsic tyrosine kinase activity that appears to be essential for many of the broad-range effects of their ligands (4, 5). Most vertebrate tissues express insulin receptors and many cell types have also IGF-I receptors.

Information on insulin action in early embryos is fragmentary. Insulin receptor mRNA and insulin-dependent tyrosine kinase activity have been well characterized in *Drosophila* embryos (6, 7), but there are no data on endogenous insulin-related molecules during *Drosophila* embryogenesis. While insulin effects on gene expression and presence of insulin-related epitopes have been demonstrated in sea urchin embryos, the insulin receptor has not been characterized (8). Preimplantation mouse embryos (9) and rat embryos in early organogenesis (10) display insulin receptors with typical ligand-binding characteristics. However, it is not known if the β subunit of these receptors is functional.

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While the paracrine/autocrine mode of action of IGF-I is fully recognized (11), insulin's nonpancreatic origin and paracrine/autocrine effects are much more controversial (12–14). A vertebrate model, the chicken embryo, allows studies from the beginning of gastrulation, throughout neurulation and organogenesis (15), stages of development hardly accessible in mammals. On the basis of immunoneutralization experiments we have postulated that insulin and insulin receptors are required for normal chicken embryo organogenesis (16). We have demonstrated that insulin mRNA and insulin are present in embryos of about 20 somites (day 2), well before the pancreas develops (17, 18). Whether the prepancreatic embryonic insulin is active is dependent upon the existence of active cell receptors capable of mediating insulin action. Binding and cross-linking techniques allowed detection of low numbers of insulin receptors as well as IGF-I receptors in day 2 embryos, when neurulation is virtually complete (19). These methods, however, lacked the sensitivity to detect the less abundant insulin receptors that we thought might be present in earlier development. Therefore, we have adapted an autoradiographic technique to whole mounts. This technique has enabled us to detect, characterize, and localize insulin receptors and IGF-I receptors during gastrulation and neurulation in chicken embryos.

We report here that the low binding of ^{125}I-labeled insulin (^{125}I-insulin) measurable during early organogenesis in the chicken reflects, indeed, presence of receptors that possess an insulin-stimulatable tyrosine kinase. The IGF-I receptor also exhibits developmentally regulated tyrosine kinase activity.

EXPERIMENTAL PROCEDURES

Embryos. Chicken embryos (White Leghorn) from stage 3–4 of Hamburger and Hamilton (15) (definitive primitive streak) to stage 14 (22 somites, neurulation completed) were studied by autoradiographic techniques. Whole blastoderms, including the extraembryonic membranes, were placed on gelatin-coated slides and their stage was determined. The slides were dried in a vacuum desiccator overnight at 4°C and stored at −70°C until binding studies were performed. For phosphorylation studies, embryos were selected on appropriate days of development and a number of embryos, from 10 at day 6 to several hundred at day 2, were pooled to obtain enough tissue for the receptor preparation.

***In Situ* Binding Studies.** Embryos were brought to room temperature before use and the slides were positioned horizontally in a tray. A silicone ring around the blastoderm held all reagents in place. Under experimental conditions (described in figure legends) the embryos were incubated in a

Abbreviations: IGF-I, insulin-like growth factor I; WGA, wheat germ agglutinin.

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total volume of 300 μl containing ^{125}I-insulin or ^{125}I-IGF-I. Characterization of binding specificity was assessed by competition curves obtained by incubating a series of similar embryos with labeled ligands and unlabeled insulin, IGF-I, or proinsulin at increasing concentrations. At the end of the incubation time the embryos were washed, air-dried, fixed, and apposed to LKB Ultrofilm ^{3}H. The autoradiograms were developed after 7–10 days and in some cases they were quantified by computer-assisted densitometry; the images were also color-coded to facilitate analysis. In addition, each embryo was scraped from the slide and collected onto paper, and the radioactivity was measured.

^{125}I-Labeled Peptide Binding to Glycoprotein-Enriched Preparations. Solubilized receptors were obtained as described (20) with minor modifications. Insulin binding and IGF-I binding experiments were performed by incubation of samples of wheat germ agglutinin (WGA)-purified preparations with 0.04–0.05 nM ^{125}I-insulin or ^{125}I-IGF-I and unlabeled peptides at increasing concentrations, as described in the legend of Fig. 5.

Exogenous Substrate Phosphorylation by Solubilized Receptors. The solubilized, WGA-purified receptors were incubated in the presence of artificial substrate, the synthetic random copolymer poly(Glu^{80}Tyr^{20}), following established protocols (21). Stimulated tyrosine kinase activity is the difference between the activities of a preparation in the presence and absence of the stimulating ligand, insulin or IGF-I at 100 nM. In some experiments, the solubilized receptors were preincubated with Fab fragments of antiserum B-10, a human polyclonal antibody to insulin receptor. The IgG fraction was purified by acid elution from staphylococcal protein-A Sepharose, and the monovalent Fab fragment was obtained by digestion with papain (50 μg/ml at 37°C for 10 hr). The reduction and alkylation were carried out with 5 mM cysteine and 10 mM iodoacetamide. This sample was dialyzed, applied to a protein-A column to remove Fc fragments, and purified by gel filtration on Sephadex G-200 to isolate Fab fragments.

Materials. ^{125}I-labeled human insulin (2000 Ci/mmol), ^{125}I-IGF-I (2000 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) were purchased from Amersham. Porcine insulin, human recombinant insulin, and bovine proinsulin were from Eli Lilly and IGF-I was purchased from AmGen Biologicals. Hepes, bovine serum albumin, bacitracin, phenylmethylsulfonyl fluoride, leupeptin, Triton X-100, *N*-acetyl-D-glucosamine, polyethylene glycol, sodium pyrophosphate, sodium orthovanadate, CTP, ATP, and synthetic tyrosine-containing polymers [poly(Glu^{80}Tyr^{20})] were purchased from Sigma. WGA coupled to agarose was from ICN–Immuno Biologicals (Lisle, IL), Ultrofilm ^{3}H was from LKB, and the developer and fixer were from Kodak. All other chemicals used were reagent grade.

RESULTS

Autoradiographic Localization of Insulin and IGF-I Binding Sites. Receptors that specifically bind insulin and IGF-I were morphologically localized in whole-mounted chicken embryos throughout gastrulation and neurulation by using *in situ* autoradiography. Autoradiograms of binding of ^{125}I-insulin (Figs. 1 and 2) and ^{125}I-IGF-I (Figs. 2 and 3) to chicken embryos showed that binding sites for both peptides were detectable in the embryo at 18 hr of development, stage 4, before the appearance of the first somite. Specific binding of each peptide per whole embryo increased with age until 52 hr of development (Figs. 1 and 3). Nonspecific binding was low until embryos reached stage 13 (50 hr). Direct autoradiographic images as well as computerized color-enhanced densitometry (Fig. 2) indicated that the highest levels of both insulin and IGF-I binding sites were in Hensen's node, neural folds, neural tube, and the developing eye. Other structures

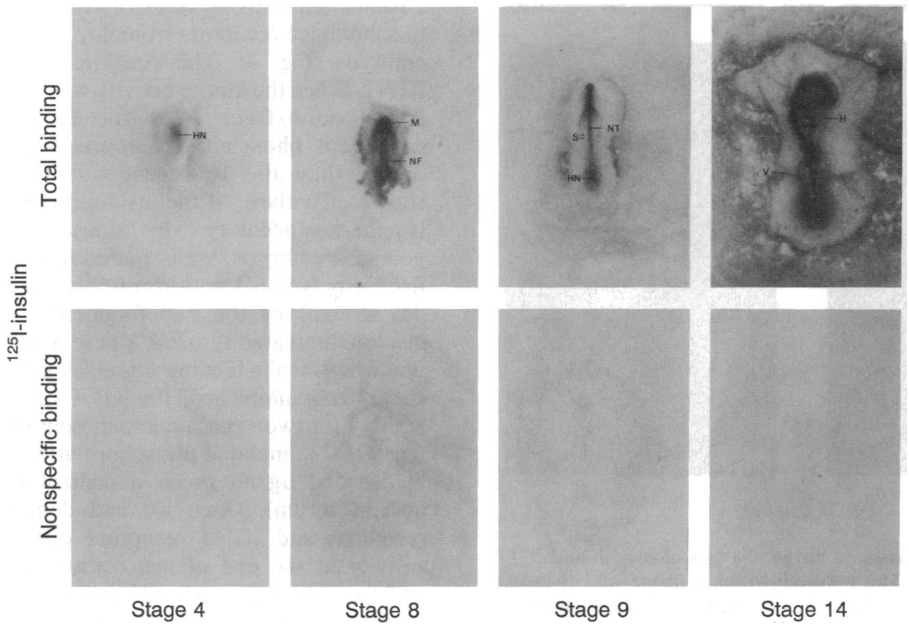


FIG. 1. *In situ* binding of ^{125}I-insulin to embryos at gastrula to postneurulation stages. Whole-mounted chicken embryos at different stages of development (ref. 15; see Fig. 3 for chronological age) were carefully removed from the yolk, transferred into cold phosphate-buffered saline (PBS, pH 7.8; Biofluids), and placed on gelatin-coated slides. The embryos were examined under a dissection microscope to assess the stage of development, and after drying they were stored at −70°C. Under experimental conditions the embryos were preincubated twice for 5 min in cold PBS (pH 7.8). Then the incubation was for 6–8 hr at 4°C in a humid chamber. The assay buffer contained 50 mM Hepes (pH 7.8), 120 mM NaCl, 15 mM $NaC_2H_3O_2$, 10 mM glucose, 2.5 mM KCl, 1.2 mM $MgSO_4$, 1 mM EDTA, bovine serum albumin (0.5%), and bacitracin (1 mg/ml). The incubation mixture (total 300 μl) included ≈30,000 cpm of ^{125}I-insulin in the absence (total binding) or presence (nonspecific binding) of unlabeled insulin at 10 μg/ml. After washing in 10 successive 1-min changes of cold PBS, the slides were air-dried quickly and fixed in a vacuum desiccator containing vapors from paraformaldehyde powder and heated at 80°C for 2 hr. The embryos were apposed to film for 1 week and photographic prints were obtained from the autoradiograms. Identical magnification (printed here ×5) and exposure length were used for all embryo stages. HN, Hensen's node; M, midbrain; NF, neural folds; S, somites; NT, neural tube; H, heart; V, vitelline vessels.

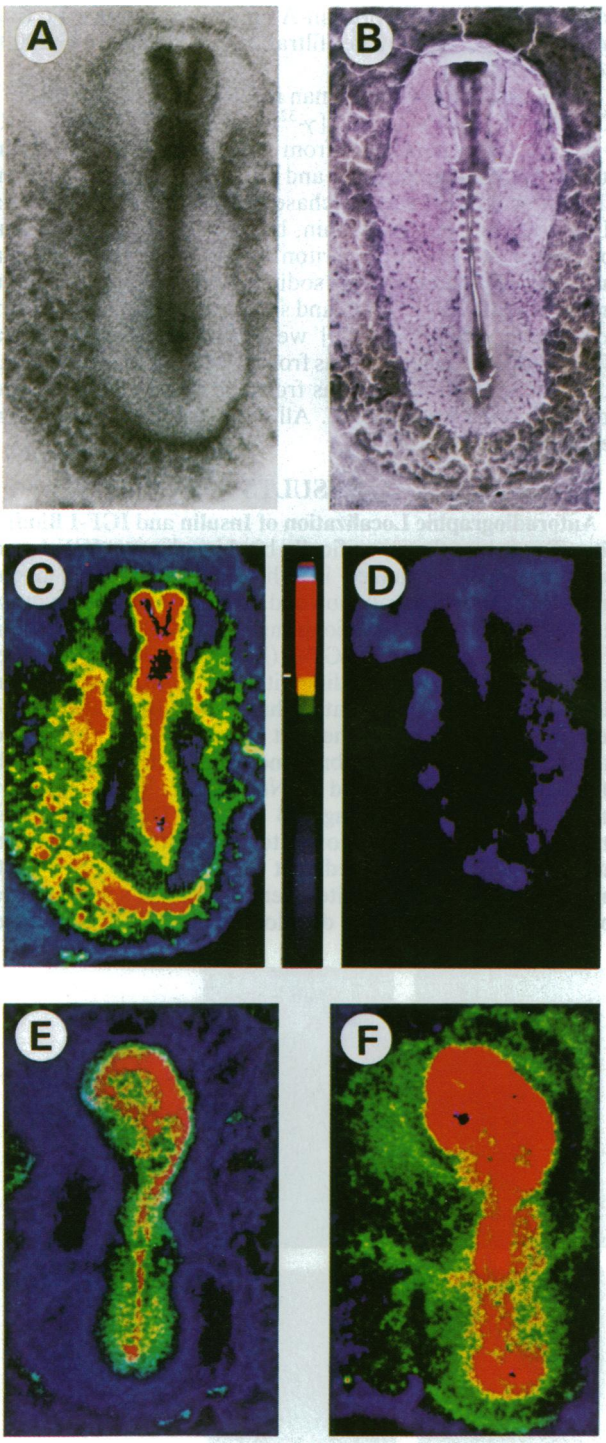
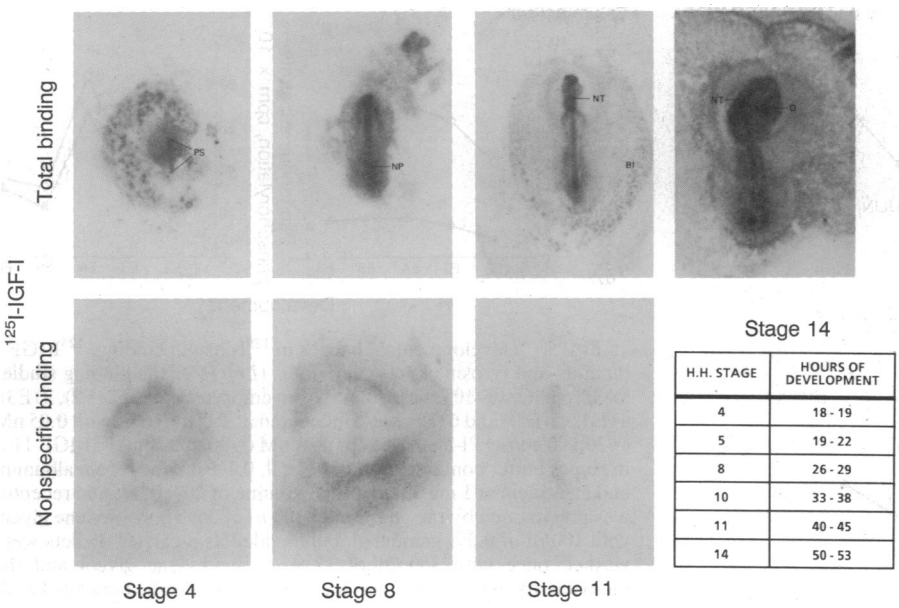


FIG. 2. Computer-assisted image analysis of insulin and IGF-I receptors in embryos. The autoradiographic image of ^{125}I-insulin binding to a stage 11 embryo (*A*; obtained as all autoradiograms presented in Fig. 1) was converted into a color-coded image (*C*) in which the highest density and, therefore, highest binding is red and the lowest is blue with intermediate densities appearing as yellow (see scale). *C* is total ^{125}I-insulin binding and *D* is nonspecific binding obtained with a similar embryo incubated with an excess of unlabeled insulin. In *B* an embryo at stage 11 of development was stained with Coomassie brilliant blue and photographed with a light microscope at ×8 (printed here ×6). The color spectrum was only moderately better for localization of receptors than the direct autoradiogram. However, in stage 14 embryos, the higher and more widespread binding of ^{125}I-IGF-I (*F*; obtained as described for *C*) than binding of ^{125}I-insulin (*E*) was better illustrated by this technique.

were only moderately labeled—e.g., the heart and the somites. Thus, ectoderm-derived structures were the richest in receptors. In contrast, the yolk sac membrane (endoderm derived) had much lower binding. The binding patterns for each peptide were almost identical, until stage 11 (≈44 hr), although the binding of IGF-I was greater with this technique, as was confirmed by direct measurement of the embryo-associated radioactivity. By stage 14 (≈52 hr), however, IGF-I binding seemed to be not only higher in general but also more uniformly widespread, particularly in cephalic structures (Fig. 2 *E* and *D*). Different levels of specific binding of each peptide to chicken embryo were related to, but clearly distinct from, the pattern of protein distribution (Fig. 2*B*).

Specificity and Scatchard Analysis of Insulin and IGF-I Binding to Whole-Mounted 40- to 45-hr Embryos. Competition studies with ^{125}I-insulin or ^{125}I-IGF-I and unlabeled peptides were performed in embryos at stage 11. ^{125}I-insulin was allowed to compete for binding with unlabeled insulin (ED_{50} = 35 ng/ml), IGF-I (ED_{50} = 125 ng/ml), or proinsulin (ED_{50} = 500 ng/ml). Binding of ^{125}I-IGF-I was displaced by unlabeled IGF-I (ED_{50} = 25 ng/ml), insulin (ED_{50} = 100 ng/ml), or proinsulin ($ED_{50} \approx 10$ μg/ml). These specificities correspond to an insulin receptor and an IGF-I receptor, respectively, although with modest higher affinity for the heterologous ligand than typical receptors from adult tissues. Scatchard analysis (not shown) showed that there were high- and low-affinity insulin receptors (curvilinear Scatchard plot) and predominant high-affinity IGF-I receptors (linear Scatchard plot).

Ligand-Stimulated Phosphorylation of Exogenous Substrate by Solubilized Receptors from Embryos at Day 2 to Day 6. Incorporation of ^{32}P into artificial substrate catalyzed by WGA-purified receptors was stimulated by 100 nM insulin in a time-dependent manner and reached a plateau at about 30–45 min (data not shown). Both insulin- and IGF-I-stimulated tyrosine kinase activities were dose dependent, in solubilized receptors from day 2 and day 4 whole chicken embryos (Fig. 4). The absolute values were higher with IGF-I. When the kinase activity was expressed relative to the *B/F* (bound/free) value for each ligand, the insulin-dependent phosphorylation was found to be remarkably higher than the IGF-I-dependent phosphorylation (not shown). To clarify if the insulin receptor was mediating the insulin-dependent tyrosine kinase activity detected in the youngest embryos, we prepared monovalent Fab fragments from a polyclonal anti-insulin receptor antibody (B-10). In day 2 embryos the Fab fragments partially inhibited the insulin-stimulated tyrosine kinase activity (up to 60% inhibition when insulin binding was also inhibited up to 50%), while they did not inhibit at all the IGF-I-stimulated tyrosine kinase activity (in two separate experiments neither IGF-I binding nor IGF-I-stimulated phosphorylation was inhibited by Fab at 200–500 μg/ml or by a control IgG preparation from normal serum). Once we had demonstrated that insulin receptors and IGF-I receptors displayed tyrosine kinase activity at the end of neurulation (2 days in the chicken embryo, which corresponds approximately to 11 days in the rat embryo and 4 weeks in a human fetus), we studied its developmental regulation during early organogenesis. In parallel, we also measured the binding of ^{125}I-insulin and ^{125}I-IGF-I by solubilized receptors prepared from whole embryos. There was a modest increase in ^{125}I-insulin binding (≈80%) and in ^{125}I-IGF-I binding (≈60%) between day 2 and day 4 of embryogenesis, with a plateau until day 6 (Fig. 5 *Left*). Surprisingly, there was a much more dramatic increase (between 5- and 10-fold in different experiments) in the ligand-dependent kinase activity of each receptor between day 2 and day 4 of development (Fig. 5 *Right* and Fig. 4).



H.H. STAGE	HOURS OF DEVELOPMENT
4	18 - 19
5	19 - 22
8	26 - 29
10	33 - 38
11	40 - 45
14	50 - 53

FIG. 3. Whole-mounted chicken embryos at different stages of development were incubated with ^{125}I-IGF-I (40,000 cpm) in the absence (total binding) or presence (nonspecific binding) of unlabeled IGF-I at 1 μg/ml with the same experimental design and conditions as described in the legend for Fig. 1. PS, primitive streak; NP, neural plate; NT, neural tube; BI, blood islands; O, optic vesicle; H.H., Hamburger and Hamilton (15).

DISCUSSION

We have localized receptors for insulin and IGF-I during gastrulation and neurulation and characterized their specificity. Since there is no blood circulation at these stages of chicken development, nor is there a pancreas (15, 22), any effects of insulin and IGF-I mediated by these receptors are conceptually paracrine effects in a "prepancreatic" embryo. The high sensitivity of the *in situ* autoradiographic approach, enhanced by computerized image analysis, has allowed demonstration of specific binding of both insulin and IGF-I at the earliest stage analyzed, the primitive streak stage (equivalent to blastula of mammals). At each embryo age, the binding of IGF-I is higher than binding of insulin, while the anatomical distribution is overlapping. Although there is widespread distribution, regions richest in insulin receptors and IGF-I receptors are of ectodermal origin. During gastrulation, there is high binding to Hensen's node, a structure implicated in the early gastrulation cell movements, equivalent to the dorsal lip of the blastopore of amphibians (23). During neurulation, high binding is found in the neural folds and neural tube and in the primitive optic vesicle, all ectodermal structures. While we do not have direct evidence of the local synthesis of insulin or IGF-I in these areas, there is insulin-related material in the day 2 chicken embryo as well as in the head region of embryos from day 3 to day 5 (17). Neurons have been reported to synthesize insulin in neonatal rabbit brain (24). IGF-I mRNA was detected in whole rat brain at embryonic day 14 (25) and found in both neuronal and glial cultures of rat fetuses (25, 26), although it was not detected in human fetal cerebral cortex (27). In addition, the chicken embryo receptors as well as the extraembryonic receptors in the yolk sac membrane may have access to the maternal insulin stored in the egg (17).

We next addressed the question of whether the insulin and IGF-I receptors in young embryos have active tyrosine kinases. We had strong indirect evidence that the insulin receptor from chicken embryos mediates developmental effects between day 2 and day 5, since anti-insulin receptor IgG (B-10) retards embryo growth in a way similar but not identical to anti-insulin IgG (28). We had also shown overall growth stimulation in embryos treated with exogenous insulin and IGF-I (29). Moreover, in explanted chicken day 1 blastoderms it has been reported that insulin increases glucose consumption (30). We purified glycoproteins from solubilized membranes, prepared from large pools of embryos throughout organogenesis, from day 2 to day 6. Embryos of all ages had measurable insulin-dependent and IGF-I-dependent tyrosine kinase activities. This indicates that the β subunit of both insulin receptors and IGF-I receptors appears to be functional [there is also ligand-dependent autophosphorylation of the β subunit of each receptor (unpublished results)]. In absolute values the two kinases were almost equipotent (Fig. 5 *Right*). Although there are more IGF-I receptors than insulin receptors present at these stages in the whole embryo, we think that insulin preferentially activates its own receptor kinase. Even at the earliest age (day 2), when insulin binding is lowest, more than 50% of the insulin-stimulated phosphorylation was inhibited by the Fab fragment of an antiserum which blocks insulin binding in this embryo and does not inhibit IGF-I binding.

The receptor-associated kinases appeared to be developmentally regulated independently of their binding activity. Between day 2 and day 4 there was a dramatic increase in kinase activity, while the increase in ligand binding by the glycoprotein-enriched preparations was small. The net gain in kinase activity of the receptors present in the embryo may be related to the beginning of tissue differentiation in some structures newly formed at this time as, for example, the cardiovascular system (circulation begins at $\approx$2.5 days), encephalic vesicles, eye lens, limb buds, etc. Very recently, ubiquitous insulin receptor transcripts have been detected in *Drosophila* throughout embryogenesis, with high levels in the developing nervous system (6). In *Drosophila*, insulin-dependent tyrosine kinase activity was also developmentally regulated (7).

In summary, our studies provide morphological mapping of insulin receptors and IGF-I receptors from gastrulation to early organogenesis in a vertebrate embryo. The overlapping distribution of the two receptors and high levels in neural structures may reflect a coordinated role of insulin and IGF-I in nervous system development. Fully stimulatable insulin receptor and IGF-I receptor kinases appear early in development and, thus, the potential involvement of these kinases in induction and differentiation events is very suggestive.

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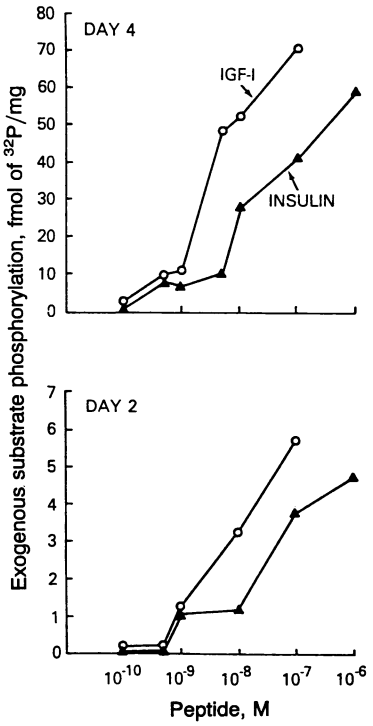

FIG. 4. Insulin and IGF-I dose-dependency of tyrosine phosphorylation by WGA-purified receptors of day 2 and day 4 embryos. Whole embryos (≈200 at day 2 and ≈50 at day 4) were homogenized and crude membranes were obtained as described (19). The membranes were solubilized in 50 mM Hepes/1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 1 kallikrein inhibitor unit/ml for up to 16 hr at 4°C. The solubilized suspensions were centrifuged at 40,000 × *g* for 1 hr and the supernatants were passed through Millipore filters (0.22 μm pores, low absorption) and immediately recycled four times over a column containing WGA coupled to agarose (20). Fractions (0.5 ml) were collected and those with the highest protein content were tested for ligand binding, pooled, and concentrated to yield a solution of about 250–400 μg/ml protein. Samples containing 10 μg of protein were incubated for 45 min at room temperature in 50 mM Hepes buffer (pH 7.6) in the presence of artificial substrate, poly(Glu^{80}-Tyr^{20}) (2 mg/ml final concentration), or buffer, with or without the addition of insulin (▲) or IGF-I (○) at various concentrations in a total volume of 140 μl. The phosphorylation reaction was allowed to proceed as described (21). Phosphorylation of endogenous substrates produced 35–50% of the total trichloroacetic acid-precipitable radioactive material in receptor preparations from embryos at day 2, and less than 12% in older embryos. This value was subtracted from the experimental points to determine the amount of radioactivity incorporated per mg of exogenous substrate.

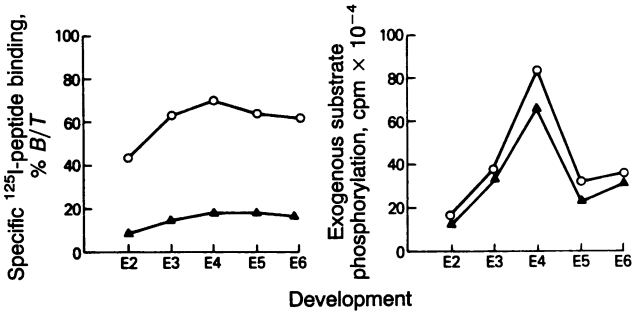

FIG. 5. Developmental changes in ^{125}I-insulin binding, ^{125}I-IGF-I binding, and tyrosine kinase activities. (*Left*) For the binding studies WGA eluates (8–10 μg of protein) from embryos at days 2 (E2), 3 (E3), 4 (E4), 5 (E5), and 6 (E6) were incubated at 4°C for 16 hr with 0.05 nM (≈30,000 cpm) ^{125}I-insulin (▲) or 0.04 nM (≈30,000 cpm) ^{125}I-IGF-I (○) in Hepes buffer containing 0.15 M NaCl, 0.1% bovine serum albumin, and bacitracin at 1 mg/ml in a total volume of 200 μl. Bound receptor was precipitated by the addition of 300 μl of 25% polyethylene glycol with 100 μl of 0.3% gamma globulin added as a carrier. Pellets were washed once with 300 μl of 12.5% polyethylene glycol and the radioactivity in the pellets was measured. The specific binding of each ligand (*B*) is expressed as percent of the total radioactivity added (*T*) after the nonspecific binding has been subtracted and is normalized to 10 μg of receptor glycoprotein. (*Right*) A similar sample of the eluates was used to measure phosphorylation of the exogenous substrate poly(Glu^{80}Tyr^{20}) as described in the legend to Fig. 4. Each ligand was used at 100 nM. Two to four independent preparations were studied at each developmental time point. Due to changes in specific activity of [γ-^{32}P]ATP between experiments, the cpm measured in the kinase assay were not directly comparable; therefore, a representative experiment is reported. The developmental changes were confirmed in other experiments. Results are expressed per 10 μg of receptor glycoprotein. The data on insulin binding are the mean values of two experiments. ○, IGF-I; ▲, insulin.

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