Insulin-like Growth Factor I and Insulin Regulate δ-Crystallin Gene Expression in Developing Lens*

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Normal development of the chicken embryo requires insulin and insulin receptors. Insulin and also insulin-like growth factor I (IGF-I) can stimulate embryonic growth when applied in vivo at the beginning of organogenesis (Girbau, M., Gomez, J. A., Lesniak, M. A., and De Pablo, F. (1987) Endocrinology 121, 1477–1482). In the present work we chose the developing eye lens, an avascular organ composed of a single cell type, to characterize further the specific effects of insulin and IGF-I upon cell differentiation and gene expression. Epithelial cells (before terminal differentiation) and fiber cells (terminally differentiated) were cultured in the presence of the hormones. δ-Crystallin mRNA steady-state levels as well as nuclear δ-crystallin gene transcription were measured. Either insulin or IGF-I (0.1–10 ng/ml) increased (2–4-fold) δ-crystallin mRNA in epithelial and fiber lens cells from day 6 embryos. The effect of insulin was largely blocked by the Fab fragment of anti-insulin receptor antibody (B-10). By contrast, as it had been shown for metabolic actions in other systems, bivalent B-10 IgG itself mimicked insulin action, i.e. it induced an increase on δ-crystallin mRNA levels. Thus, insulin appears to act through its own receptor in regulating the levels of δ-crystallin mRNA. There was a differential transcriptional component in insulin and IGF-I effects on δ-crystallin gene expression. IGF-I induction of transcription, as measured by nuclear run-on assay, is greater than insulin induction (≈2.5-fold versus 1.4-fold) and faster. The δ-crystallin gene will provide the opportunity to analyze the action of insulin and IGF-I on the expression of a structural protein marker of cell differentiation during early embryonic development.

The interaction of insulin and insulin-like growth factor I (IGF-I) with their cell surface receptors initiates metabolic, growth, and differentiation responses in multiple cell types. Insulin, which has been studied best, can influence those cellular processes by mechanisms which include regulation of gene expression at transcriptional and posttranscriptional levels. Both negative and positive transcriptional regulation by insulin have been reported for a few enzymes (1–3). In addition, insulin regulates mRNAs which are associated with changes in cell growth and differentiation (4, 5). Although the information is limited, IGF-I has been reported to regulate mRNAs both positively (6) and negatively (7).

δ-Crystallin is the major structural protein of the lens in chicken embryogenesis, accounting for more than 70% of total cell protein and mRNA (8). Its expression is a specific marker of differentiation of the lens epithelial cells into fibers. Accumulation of δ-crystallin mRNA has been found to be stimulated by high concentrations of insulin as well as by fetal calf serum (9). In this system, IGF-I was more potent than insulin-promoting cell elongation (10), a reflection of morphological cell differentiation. The effect of IGF-I on δ-crystallin mRNA, however, has not been studied.

Since we had previously found that both epithelial cells and terminally differentiated fiber cells display insulin receptors and IGF-I receptors (11), we undertook the characterization of the effects of both peptides at low concentrations in the two cell populations of the developing lens. Our goal was to study the action of insulin and IGF-I upon expression of a gene during early embryonic development. Additionally, we wanted to clarify whether the effect of insulin was mediated through the insulin receptor.

We show that insulin, as well as IGF-I, at nanomolar concentrations stimulates the expression of δ-crystallin mRNA in both epithelial cells and fully differentiated fibers. We demonstrate that the insulin receptor is involved in this action of insulin. The effects of IGF-I and insulin on δ-crystallin mRNA are, at least partially, transcriptional. Further, the IGF-I effect on transcription is more potent and faster than insulin’s effect.

EXPERIMENTAL PROCEDURES

Cell Culture—Fertile chicken eggs were obtained from Truslow Farms (Chester, MD) and incubated at 38 °C and 60–90% relative humidity. Lenses from day 6 embryos (hatching is at day 21) were removed and the epithelia and fibers microdissected (Fig. 1A) according to established methods (8). The two cell populations were transferred separately to 35-mm culture dishes containing warm F-10 Ham’s medium (GIBCO). Incubation was continued for different times as specified in a cell culture incubator at 37 °C, 5% CO2, with or without the addition of recombinant human peptides, insulin (Lilly), IGF-I (AmGen Biologicals, Thousand Oaks, CA), proinsulin (Lilly) or IGF-II (Bachem Inc., Torrance, CA).

Total RNA Extraction and Blotting—After 24 h of incubation under standard conditions, cells were homogenized in 10 mM Tris, 140 mM NaCl, 1% sodium dodecyl sulfate, and the total cellular RNA was extracted as described by Davis et al. (12). An initial phenol/chloroform (1:1) extraction was followed by a second chloroform extraction. The total RNA was precipitated with 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol. After centrifugation the pellet was dried and resuspended in 100 μl of RNase-free water. To detect the δ-crystallin mRNA, we used as probe a 626-base pair BglII fragment of pEWC clone of a chicken δ-crystallin cDNA (kind gift of E. F.
RESULTS

δ-Crystallin-mRNA Is Expressed in Epithelial and Fiber Lens Cells—At day 6 of development, the chick embryo lens expresses a significant amount of δ-crystallin mRNA in vivo. When the epithelial cells were microdissected from the large fiber mass and the levels of δ-crystallin mRNA were estimated for each cell type the expected difference was observed (Fig. 1).
IGF-I and Insulin Regulate \( \delta \)-Crystallin mRNA

**TABLE I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Epithelial cells</th>
<th>Fiber cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proinsulin (10 ( \mu )g/ml)</td>
<td>1.45 ± 0.2</td>
<td>0.87 ± 0.1</td>
</tr>
<tr>
<td>IGF-II (10 ( \mu )g/ml)</td>
<td>1.82 ± 0.7</td>
<td>1.75 ± 0.5</td>
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**Fig. 2.** \( \delta \)-Crystallin mRNA levels after treatment with insulin (C—C) and IGF-I (O—O). Cells were incubated for 24 h without or with concentrations of the hormones from 0.1 to 10 ng/ml. Total RNA was prepared, loaded into a nylon membrane, and this hybridized to the \( 32^P \)-labeled cDNA for \( \delta \)-crystallin. The signal obtained was quantitated by densitometry and normalized after rehybridizing the slot-blot with a 28 S rRNA probe. The increase in \( \delta \)-crystallin mRNA relative to the level in untreated cultures is shown in an arbitrary scale. The mean values of four experiments and the standard error are represented. When high insulin concentrations (1 \( \mu \)g/ml) were added to the cultures the results obtained were very variable. We had evaluated the effect of a 24-h incubation with 1 \( \mu \)g/ml of insulin upon specific \( ^{125} \)I-insulin binding in the lens epithelial cells and showed a marked receptor down-regulation (11).

**Fig. 3.** Anti-insulin receptor (B-10) IgG mimics insulin effect on \( \delta \)-crystallin mRNA levels. Each value represents the mean ± S.E. of two separate experiments in which duplicate dishes with cells from five lenses in each were cultured. The stimulation observed here is very small when compared with the effect of insulin and IGF-I (Fig. 2).

The \( \delta \)-crystallin mRNA signal was many fold higher in the fully differentiated fiber cells. Although low, the \( \delta \)-crystallin mRNA levels were nevertheless measurable in the mitotic epithelial cells, which probably included a population of "equatorial" partially differentiated cells. The specificity of the probe was confirmed by the finding of a single transcript present in eye but not detected in liver and brain in a gel-fractionated RNA blot (Fig. 1B).

Stimulatory Effect of Insulin or IGF-I on \( \delta \)-Crystallin mRNA Levels—When cells were incubated with IGF-I or insulin for 24 h, there was an increase in steady-state levels of \( \delta \)-crystallin mRNA, in epithelial and fiber cells (Fig. 2). After hybridization with the \( \delta \)-crystallin probe, each slot-blot was washed and rehybridized with a cDNA probe for 28 S rRNA. The signal obtained by densitometry was used as normalization factor to quantitate the specific effect of the hormones on \( \delta \)-crystallin, independent of possible increases in total RNA and to correct for differences in RNA loading. IGF-I induced some increase in 28 S rRNA levels. Therefore, even if the absolute signal obtained in slot-blot was higher in IGF-I treated cells, after normalization insulin appeared to be slightly more potent than IGF-I increasing specifically \( \delta \)-crystallin mRNA.

Analog Specificity Supports Mediation of Hormonal Action by Two Receptor Types—The similar dose-response curves obtained when epithelial and fiber cells were treated with either insulin or IGF-I (Fig. 2) suggested that the effect of \( \delta \)-crystallin mRNA levels was mediated by each peptide through its own receptor. In addition, in epithelial, proinsulin was approximately 15% as potent as insulin increasing \( \delta \)-crystallin mRNA, and IGF-II was 20% as potent as IGF-I (Table I and Fig. 2). Although the stimulation caused by the analogs was marginal, these potencies are compatible with the affinities of proinsulin for the insulin receptor and of IGF-I for the IGF-I receptor, respectively. In fiber cells, where insulin receptors are much less abundant than IGF-I receptors, proinsulin's effect was undetectable while IGF-II had a small stimulatory effect (Table I).

**Antibody Studies Indicate the Involvement of the Insulin Receptor on \( \delta \)-Crystallin mRNA Regulation**—The effect of insulin on \( \delta \)-crystallin mRNA accumulation appeared to be largely mediated through the insulin receptor. We have exploited the dual capacity of antireceptor antibodies either to mimic (bivalent fragments) or to inhibit (monovalent fragments) insulin action when they bind to the insulin receptor.

When the cell were incubated for 24 h only in the presence of 20 or 200 \( \mu \)g/ml of antiserum B-10 bivalent IgG (insulin-free preparation), an insulin-mimicking effect was observed, with an increase in \( \delta \)-crystallin mRNA levels, of 2–2.5-fold over control (Fig. 3). By contrast, when the cells were incubated with both insulin (10 ng/ml) and the monovalent Fab fragment (200 \( \mu \)g/ml) of B-10, the effect of the peptide was largely blocked by the antiserum and not by a control Fab fragment. Up to 95% of the insulin-stimulated accumulation of \( \delta \)-crystallin mRNA was inhibited in the epithelial cells (mean inhibition in two experiments was 85 ± 14%) and up to 50% in fibers (mean inhibition in three experiments was 44 ± 6%). Fig. 4 shows a representative slot-blot of this experiment, hybridized with the \( \delta \)-crystallin and control probes. This concentration of B-10 Fab did not inhibit IGF-I binding while it inhibited up to 50% of the insulin binding to solubilized receptors from whole day 2 chicken embryos (23).
INSULIN + B-10Fab

FIG. 4. Anti-insulin receptor (B-10) Fab fragments inhibit insulin effect on δ-crystallin mRNA levels. Fiber cells (representative slot-blot shown) and epithelial cells were incubated under standard conditions for 24 h with 10 ng/ml of insulin alone or with insulin plus 200 μg/ml of B-10 Fab fragments. Levels of δ-crystallin mRNA were estimated as described in the legend for Fig. 2. After stripping off the δ-crystallin probe, the filters were rehybridized with a human 28 S rRNA cDNA probe, to assess variations in loading and the possible general effect on ribosomal RNA levels (observed in IGF-I-treated cells). Each filter was quantitated for δ-crystallin mRNA levels, normalizing the signal relative to the 28 S RNA values.

Fig. 5. Nuclear transcription of δ-crystallin is stimulated by IGF-I and insulin. Isolated nuclei from untreated or treated epithelial and fiber cells were incubated in vitro with [α-32P]UTP to allow elongation of nascent transcripts. The labeled RNA was hybridized to a nitrocellulose filter where oligomers complementary (antisense) or identical (sense) to a region of δ-crystallin mRNA were bound. The inset shows a representative experiment of fiber cells incubated for 1 h in the absence of hormones (control) or in the presence of 10 ng/ml of IGF-I. When the incubation also included 2 μg/ml of α-amanitin, the signal was erased, indicating that it was the result of RNA polymerase II transcription. The main figure shows the time-course of transcriptional effect of insulin (○—○) and IGF-I (●—●). Epithelial and fiber cells were incubated for various periods of time (1.5, or 24 h) in the absence (control) or presence of 10 ng/ml of either insulin or IGF-I. Nuclei were isolated at the end of the incubation and transcription was allowed to continue in vitro as described under "Experimental Procedures." The relative increase in transcription is represented using an arbitrary scale, considering the control, untreated cells, as 1. We have pooled the results of epithelial and fiber cells because they were very similar. The mean and standard error for experiments done with independent nuclear preparations at each time point is represented (at 1 h, n = 12; at 5 h, n = 6; at 24 h, n = 8).

message RNA processing, transport, and stabilization. To assess the transcriptional component of this regulation, we used a nuclear run-on assay optimized for small amounts of our primary embryonic tissue (17). Data obtained using this transcription assay with isolated nuclei from either epithelial or fiber cells, incubated in the presence or absence of the peptides, were consistent with regulation of δ-crystallin transcription by insulin and IGF-I (Fig. 5). IGF-I appears to have a greater and faster effect on transcription of this gene than insulin. When the incubation of nuclei was carried out in the presence of 10 ng/ml of IGF-I with the addition of 2 μg/ml of α-amanitin, an inhibitor of RNA polymerase II (18), transcription of δ-crystallin was blocked both in basal and stimulated conditions (Fig. 5, inset).

DISCUSSION

During development, growth and cell differentiation are likely to be regulated by some of the well-known growth factors (19, 20). Since insulin receptors and IGF-I receptors are widespread in embryos of mammals (21, 22) and chicken (23) as well as insulin receptors in Drosophila (24) before organogenesis begins, insulin and IGF-I may have specific roles in the regulation of expression of genes important in tissue differentiation, in addition to their vital function in growth. General stimulation of protein synthesis has been reported in Xenopus oocytes microinjected with insulin (25) and in insulin-treated embryos of mouse (26) and chicken (27).

We have now chosen to study one of the best defined developmental systems, the chicken embryo eye lens, where a single cell type is represented in two stages of differentiation: the epithelial, mitotic cells and the terminally differentiated fibers, specialized in the synthesis of crystallins. Insulin probably has multiple effects in the lens cells as it does in most cell types. It increases amino acid uptake (9). In addition, in the absence of changes in cell number, 10 ng/ml to 1 μg/ml of insulin, as well as vitreous humor and serum, have been reported to promote morphological differentiation of lens epithelial cells. Interestingly, cell elongation is stimulated by insulin to the same maximum as by 15% of fetal calf serum, and the effects are not additive (28). Low concentration of IGF-I was also effective causing cell elongation and has been proposed to be the physiological local regulator of this differentiation process (10). While IGF-I mRNA has been detected in human sclera and cornea (29) and we have detected insulin mRNA in chick embryo nonpancreatic tissues (30), we have not detected insulin mRNA in chick embryo whole eye by RNA filter analysis.3

In this primary culture system, we show that insulin and IGF-I, each acting largely through its own receptor, regulate δ-crystallin gene expression. Both peptides increase the steady-state levels of δ-crystallin mRNA (independently of the effect on rRNA) in epithelial and fiber cells. The little effect of IGF-I at low concentrations in fibers, compared with insulin's effect, may be due to an ability of insulin to maintain the general health of these cells (generally maintained poorly in culture), in addition to its specific action on δ-crystallin.

The effective concentration of the peptides used in the present study suggested that each could be acting through its own specific receptor. To further clarify this aspect, in the case of insulin we used a polyclonal anti-insulin receptor antisemur (B-10) extensively characterized in chick embryo (31) as well as other systems (32). "Short-term" (32) and "long-term" (14) actions of insulin had been reported to be triggered by this antisemur. Using bivalent IgG molecules we could mimic the effect of insulin on δ-crystallin mRNA accumulation in epithelial and fiber cells. Our finding is the first report of an effect of B-10 in cell differentiation and gene expression. This result indicated that the insulin recep-

3 By run-on transcription assay there was low level of transcription of both strands of the chicken insulin gene in epithelial and fiber lens cells. The significance of these nuclear transcripts is unknown (P. Zelenka and F. de Pablo, unpublished observations).
tors present in epithelial cells and even the low number present in fiber cells (11) are indeed functional at postbinding steps. By contrast, to test directly if the effect of insulin treatment could be blocked by the anti-insulin receptor antibody we prepared monovalent Fab fragments and added them to the culture simultaneously with the hormone. The inhibition of the effect of insulin was larger in epithelial cells suggesting that insulin acts predominantly through the insulin receptor. In fiber cells it remains possible that both insulin and IGF-I receptors are involved. Presumably IGF-I is acting through the IGF-I receptors, present in significant number in both epithelial and fiber cells. Unfortunately, the lack of a suitable antibody cross-reacting with the chicken IGF-I receptor did not allow us to extend the blocking experiments to the effect of IGF-I as well.

The time course and maximal effect on transcription of the &-crystallin obtained in a nuclear run-on assay was different for insulin and IGF-I. With IGFI the peak (≈2.5-fold induction) was reached at 1 h while insulin's maximal stimulation (≈1.4-fold) was after several hours of incubation. Neither were effective after 24 h. This may be a reflection of the involvement of separate insulin receptors and IGF-I receptors or, perhaps, that the overall mechanism of regulation of &-crystallin gene expression by the two hormones is different. Since steady-state levels of mRNA measured at 24 h were similar after treatment with either hormone, the results suggest an additional posttranscriptional effect of insulin, at the level of mRNA processing, transport, or stabilization. Differential regulation of prolactin mRNA levels by insulin, stimulatory, and by IGF-I, unresponsive, has been reported (34). We must emphasize that from the results presented in this paper it is not clear whether the transcriptional effect of insulin and IGF-I is specific for &-crystallin or it reflects a more general effect on RNA polymerase II-mediated transcription. Nor is it conclusively proven whether the transcriptional component of insulin action is itself mediated through the insulin receptor, or only the posttranscriptional component. However, ongoing experiments in which we are transfecting epithelial cells with hybrid &-crystallin/chloramphenicol acetyltransferase gene constructs (39) have confirmed the specificity of the effect of IGF-I and insulin on the &-crystallin promoter and not other promoters.4 We are presently attempting the identification of any insulin and IGF-I-responsive elements which may be present in the 5'-upstream region of the &-crystallin gene. Eventually this approach may permit us to identify transacting factors which could be involved in insulin and IGF-I action at the gene level during development.

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References


* J. Alemany, T. Borras, and F. de Pablo, manuscript in preparation.