

# Fibroblast Growth Factor Receptor Levels Decrease during Chick Embryogenesis

Bradley B. Olwin\* and Stephen D. Hauschka‡

\*Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706; and ‡Department of Biochemistry, University of Washington, Seattle, Washington 98195

**Abstract.** Two putative receptors for fibroblast growth factor (FGF) of ~150 and 200 kD were identified in membrane preparations from chick embryos. Specific binding (femtomoles/milligram) of <sup>125</sup>I-aFGF to whole chick embryonic membranes was relatively constant from day 2 to 7, then decreased fivefold between days 7 and 13. Day-19 chick embryos retained <sup>125</sup>I-aFGF binding at low levels to brain, eye, and liver tissues but not to skeletal muscle or cardiac tissues. The 200-kD FGF receptor began to decline between day 4.5 and 7 and was barely detectable by day 9, whereas the 150-kD FGF receptor began to decline by day 7 but was still detectable in day-9 embryonic membranes. It is not known whether the two FGF-binding proteins represent altered forms of one polypeptide, but it is

clear that their levels undergo differential changes during development. Because endogenous chick FGF may remain bound to FGF receptor in membrane preparations, membranes were treated with acidic (pH 4.0) buffers to release bound FGF; such treatment did not affect <sup>125</sup>I-aFGF binding and moderately increased the number of binding sites in day-7 and -19 embryos. Consequently, the observed loss of high affinity <sup>125</sup>I-aFGF binding sites and FGF-binding polypeptides most likely represents a loss of FGF receptor protein. These experiments provide in vivo evidence to support the hypothesis that regulation of FGF receptor levels may function as a mechanism for controlling FGF-dependent processes during embryonic development.

**G**ROWTH factors mediate crucial regulatory events governing embryonic development. Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively)<sup>1</sup> have been shown to induce mesoderm tissue in *Xenopus* animal region explants (Slack et al., 1987; Kimelman and Kirschner, 1987) and repress skeletal muscle differentiation in human, mouse, and chick skeletal muscle myoblast cultures (Linkhart et al., 1981; Clegg et al., 1987; Seed and Hauschka, 1988; Snow, M. H., and S. D. Hauschka, unpublished observations). Fibroblast growth factor (FGF) effects in vivo include modulation of mesoderm and endoderm differentiation in rat embryos (Liu and Nicoll, 1988) and induction of angiogenesis (reviewed in Baird et al., 1986; Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1987). FGF-like polypeptides have been identified in unfertilized eggs and embryos from *Xenopus* (Kimelman et al., 1988) and chick (Seed et al., 1988). FGF has also been identified in the chick limb bud (Seed et al., 1988; Muniam et al., 1988) and brain (Risau, 1986; Risau et al., 1988; Mascarelli et al., 1987) and has been purified from adult chick skeletal muscle (Kardami et al., 1985). Immunolocalization studies have shown that bFGF is present in the developing cardiac

and somite regions of chick embryos as early as stages 12 and 19, respectively (Joseph-Silverstein et al., 1989). The immunostaining persists in later heart muscle and in limb myoblasts and myotubes through day 12 (Joseph-Silverstein et al., 1989). Immunopositive bFGF staining has also been observed in adult chick muscle fiber basement lamina, but not on the muscle fibers (DiMario et al., 1989).

The FGF-dependent induction of mesoderm in *Xenopus* animal pole explants (Slack et al., 1987; Kimelman and Kirschner, 1987) and repression of terminal differentiation in chick skeletal muscle cultures (Seed and Hauschka, 1988) suggests the presence of FGF receptors in these tissues. FGF receptors have been identified in many cultured primary cells and cell lines (for reviews see Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1987); two studies of FGF receptors in vivo have reported their presence in membranes from adult bovine brain (Courty et al., 1988) and murine embryos (Olwin and Hauschka, 1989).

The present studies were undertaken to identify embryonic chick FGF receptors and to quantitate the levels of FGF receptor during chick development. Previous experiments had demonstrated that FGF receptors are present at much greater levels in embryonic vs. adult murine tissues (Olwin and Hauschka, 1989). Since chick embryos had been shown to contain high levels of FGF (Seed et al., 1988), we anticipated that embryonic chick tissues would exhibit detectable

1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; PBSMG, PBS containing 2 mM MgCl<sub>2</sub> and 0.2% bovine gamma globulin.

levels of FGF receptor. In addition, the disappearance of growth factor receptors during skeletal muscle differentiation in culture (Lim and Hauschka, 1984; Clegg and Hauschka, 1987; Olwin and Hauschka, 1988) suggested that FGF receptor levels were likely to undergo changes during chick embryogenesis. The data presented in this report provide preliminary confirmation of these predictions.

## Materials and Methods

Embryonic chick tissue was obtained from White Leghorn eggs incubated in a forced-draft incubator. Na<sup>125</sup>I and [<sup>3</sup>H]thymidine were purchased from New England Nuclear (Boston, MA). Disuccinimidyl suberate was purchased from Pierce Chemical Co. (Rockford, IL) and HVLP Durapore membranes were purchased from Millipore Continental Water Systems (Bedford, MA). aFGF and bFGF were purified from bovine brain as previously described (Olwin and Hauschka, 1986, 1989) by heparin-agarose affinity chromatography and ion exchange chromatography on mono-S columns (Pharmacia Fine Chemicals, Piscataway, NJ). Purity of aFGF and bFGF preparations was >95% as judged by reverse-phase HPLC analysis on a Vydac C<sub>4</sub> column and by silver staining of FGF preparations analyzed by SDS-PAGE (Laemmli, 1970).

### Iodination of aFGF and bFGF

A procedure was developed using chloramine T that allows iodination of aFGF and bFGF to high specific activity with full retention of biological activity and recoveries of FGF between 30 and 60%. To 0.5 μg of either aFGF or bFGF, 10 μl of 0.5 M sodium phosphate, pH 7.0, 15 μl of 0.45 M chloramine T, and 0.5 mCi of Na<sup>125</sup>I were added for a final volume of 50 μl. The reaction was allowed to proceed for 2 min, and 50 μl of 0.025 M DTT was added and allowed to react for 3 min. The <sup>125</sup>I-FGF was then diluted with 100 μl of 20 mM Hepes, pH 7.4, and 0.2% bovine gamma globulin and purified on an 0.2-ml heparin-agarose column as described previously (Olwin and Hauschka, 1986). Addition of DTT increased recoveries from heparin-agarose columns by 1.5- to 2-fold (Kan et al., 1988). <sup>125</sup>I-FGF was then diluted to 5 × 10<sup>5</sup> cpm/μl and stored at 4°C. The labeled proteins were stable for at least 1 mo, and specific activities, determined from MM14 cell growth assays (Olwin and Hauschka, 1986), ranged from 3,000 to 7,000 dpm/fmol.

### Embryonic Membrane Preparation

Chick developmental stages were determined according to Hamburger and Hamilton (1951). Data were obtained from chick embryos at stages 15, 18, 20, 25, 29, and 31, which correspond to embryonic days 2, 3, 3.5, 4.5, 6, and 7, respectively. Older chick embryos were not staged. In the text, embryos will be identified by their age in days. Unless otherwise described, embryonic chick cell membranes were prepared by dissection of embryos from the surrounding extraembryonic membranes and yolk, washing the embryos three times with PBS (50 mM sodium phosphate, pH 7.4, and 100 mM NaCl), adding 2 vol of homogenization buffer (20 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 20 kallikrein units aprotinin/ml, 1 μg/ml leupeptin, 1 mM PMSF), and homogenizing three times for 30-s bursts at 10,000 rpm with a tissue homogenizer (Tekmar Co., Cincinnati, OH). The homogenate was then centrifuged at 15,000 g for 45 min, resuspended in 2 vol of homogenization buffer by homogenization, and centrifuged at 100,000 g for 30 min. The pellet was resuspended in 2 vol of homogenization buffer and centrifuged twice more as described above. Final protein concentrations were adjusted to 5–10 mg/ml, and crude membranes were immediately frozen in liquid N<sub>2</sub> and stored at -70°C. No loss in receptor affinity or number was observed upon freezing and storage at -70°C for 2 wk.

### Membrane FGF Receptor Assay

Particulate FGF receptor was assayed as follows. Membrane protein (20 μg) in PBS containing 2 mM MgCl<sub>2</sub> and 0.2% bovine gamma globulin (PBSMG) was added to tubes containing <sup>125</sup>I-aFGF and <sup>125</sup>I-bFGF with or without a 100-fold molar excess of unlabeled aFGF or bFGF and PBSMG for a final volume of 0.1 ml. After 30 min at 22°C, the assays were terminated by dilution with 2.5 ml PBSMG and immediate application under vacuum to Durapore HVLP membrane filters (25 mm, 0.45 μm; Millipore Contin-

ental Water Systems) presoaked in 5% bovine gamma globulin. The tubes were rinsed twice with 2.5 ml PBSMG. The filters were then removed, and the <sup>125</sup>I-aFGF or <sup>125</sup>I-bFGF bound to the particulate membrane fraction was determined by counting in a gamma counter. Less than 0.2% of the total applied <sup>125</sup>I counts per minute bound to filters in the absence of membranes.

### Cross-linking <sup>125</sup>I-aFGF Membrane Receptors

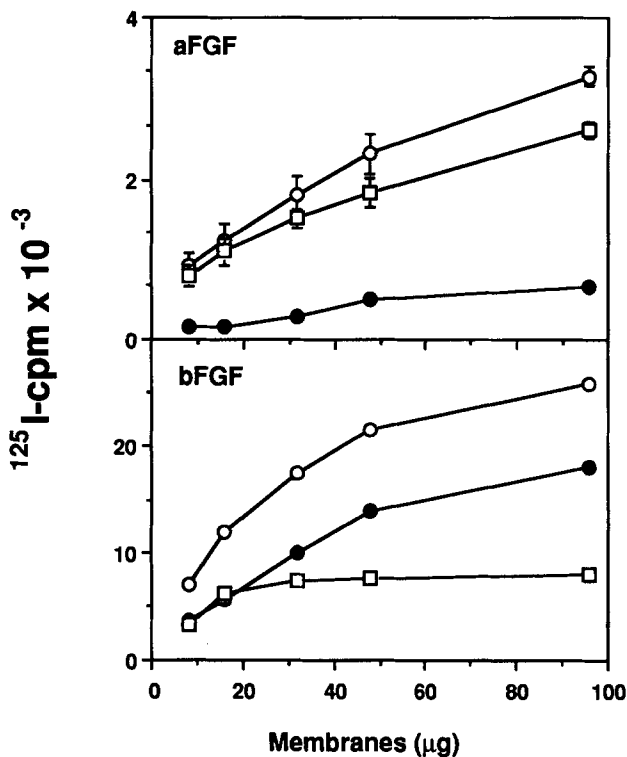
Cross-linking of <sup>125</sup>I-aFGF to particulate FGF receptors was accomplished by performing a binding assay with the following modifications and additions. Membranes (50 μg per assay) were diluted 10-fold with PBS after the 30-min incubation for <sup>125</sup>I-aFGF binding, centrifuged 5 min at 13,000 g, and washed once with PBS. The pellet was then resuspended in 1 ml of PBS containing 0.15 mM disuccinimidyl suberate (diluted from an 0.05 M stock in DMSO) and incubated at 22°C for 15 min followed by addition of 1 μl of 1 M unbuffered Tris base. The reaction was centrifuged as above, resuspended in 25 μl homogenization buffer containing 1% Triton X-100, and incubated on ice for 15 min; insoluble materials were removed by centrifugation as above. The supernatant was diluted 1:1 in H<sub>2</sub>O, SDS-PAGE sample buffer was added, and the samples were boiled for 2 min. Samples were analyzed by SDS-PAGE in 7.5% gels and subjected to autoradiography at -70°C with a Cronex Lightning Plus intensifying screen (DuPont Co., Wilmington, DE). Protein concentrations were determined according to Bradford (1976) using Pentex BSA (Miles Scientific Div., Naperville, IL) as a standard.

## Results

### Identification and Characterization of Chick FGF Binding Sites

Chick FGF receptors were detected by incubating chick membranes with <sup>125</sup>I-aFGF and <sup>125</sup>I-bFGF. Both labeled ligands exhibit high affinity, specific binding to chick embryonic membranes (Fig. 1). Isolation of plasma membranes by discontinuous gradient centrifugation yielded similar results to those observed in Fig. 1, with a 40% recovery of <sup>125</sup>I-aFGF binding sites (our unpublished data). Because 60% losses of <sup>125</sup>I-aFGF binding occur upon isolation of plasma membranes, unfractionated membrane preparations were used for subsequent experiments. <sup>125</sup>I-aFGF binding exhibits much less low affinity and nonspecific binding than does <sup>125</sup>I-bFGF (Fig. 1). The results from Fig. 1 suggested that 20 μg of membrane protein was an optimum for the binding analyses. In addition, these data demonstrate saturation of <sup>125</sup>I-aFGF and <sup>125</sup>I-bFGF by FGF-binding proteins in the membrane preparations. The high levels of "nonspecific" binding of bFGF are not due to contaminants in the bovine bFGF preparation since recombinant human <sup>125</sup>I-bFGF yields essentially identical results (our unpublished data). Attempts to reduce nonspecific and low affinity <sup>125</sup>I-bFGF binding by washing membranes with 1–2 M NaCl reduced both the total and nonspecific binding by 5- to 10-fold (our unpublished data). To avoid potential artifacts due to high levels of <sup>125</sup>I-bFGF nonspecific binding, the remainder of these studies examine only <sup>125</sup>I-aFGF binding. Although aFGF and bFGF compete for the same high affinity receptors in a variety of vertebrate cell lines (Olwin and Hauschka, 1986, 1989; Neufeld and Gospodarowicz, 1986), distinct bFGF receptors or receptors specific for chick FGF present in the chick embryo might not be recognized by <sup>125</sup>I-aFGF; if so, the total FGF receptor number may be underestimated.

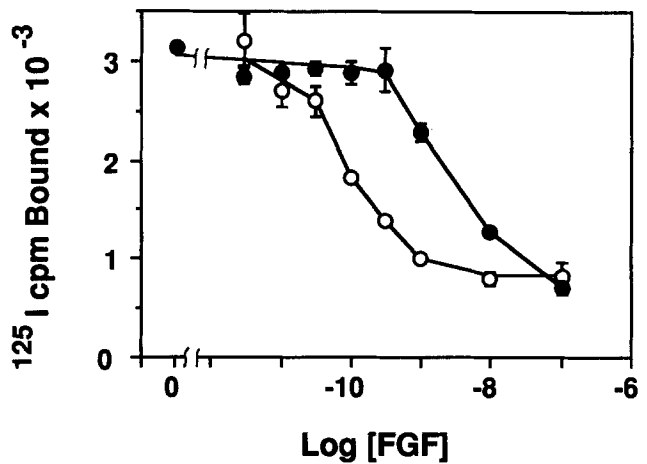
Dissection of day-7 chick embryos revealed nearly equivalent specific binding of <sup>125</sup>I-aFGF in embryonic head, body, and limbs (our unpublished data). Since equivalent specific



**Figure 1.** Binding of bovine  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF to embryonic membranes from day-7 chick embryos. (*Top*) Increasing amounts of crude chick membranes were incubated with 200 pM  $^{125}\text{I}$ -aFGF in the absence ( $\circ$ ) and presence ( $\bullet$ ) of a 100-fold molar excess of aFGF and processed as described in Materials and Methods. Data are plotted as the mean and standard deviation of background subtracted  $^{125}\text{I}$  counts per minute. Specifically bound  $^{125}\text{I}$ -aFGF ( $\square$ ) was obtained by subtraction of  $^{125}\text{I}$ -aFGF counts per minute bound in the presence of excess aFGF from the total  $^{125}\text{I}$ -aFGF counts per minute bound. (*Bottom*) Increasing amounts of crude chick membranes were incubated with 200 pM  $^{125}\text{I}$ -bFGF in the absence ( $\circ$ ) and presence ( $\bullet$ ) of a 100-fold molar excess of bFGF. Data were plotted and specifically bound  $^{125}\text{I}$ -bFGF ( $\square$ ) was determined as described above.

binding of  $^{125}\text{I}$ -aFGF was observed in these three regions, whole chick embryos were used for the initial analysis of FGF receptor expression during early stages of chick development. A survey of individual tissues was examined in chick embryos greater than 7-d-old since preparation of whole chick embryo membranes was not feasible.

Unlabeled aFGF and embryonic chick FGF (Seed et al., 1988) compete for specific binding of  $^{125}\text{I}$ -aFGF, suggesting these factors bind to common binding sites (Fig. 2). Competition of bound  $^{125}\text{I}$ -aFGF was expected to require more chick FGF than bovine aFGF since chick FGF was not purified to homogeneity (Seed et al., 1988). Because chick FGF and bovine aFGF both displaced 82% of the total bound  $^{125}\text{I}$ -aFGF, bovine  $^{125}\text{I}$ -aFGF appears to interact with at least one class of chick FGF receptors. Moreover, a purified 150-kD chick FGF receptor binds bovine aFGF, human recombinant bFGF, and bovine bFGF with high affinity (Burrus and Olwin, 1989). Consequently, it seemed reasonable to use bovine  $^{125}\text{I}$ -aFGF for further quantitation and identification of chick FGF receptors during chick embryogenesis.

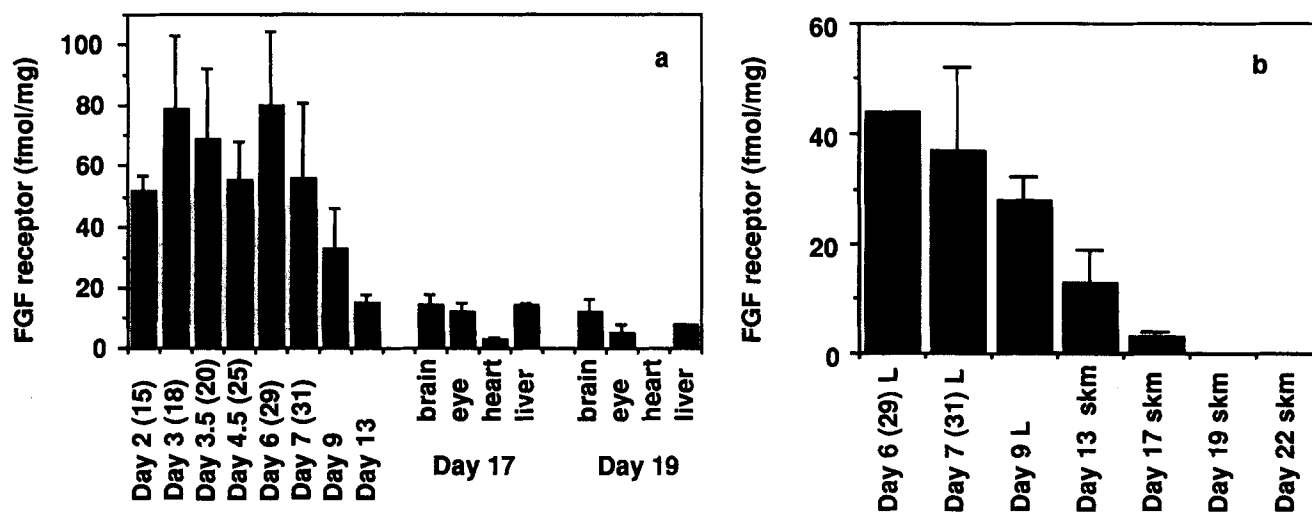


**Figure 2.** Competition of bound bovine  $^{125}\text{I}$ -aFGF by bovine aFGF and chick FGF. Crude chick membranes (20  $\mu\text{g}$ ) from day-7 embryos were incubated with 75 pM  $^{125}\text{I}$ -aFGF in the presence of increasing concentrations of aFGF ( $\circ$ ) or partially purified chick FGF ( $\bullet$ ) and specifically bound  $^{125}\text{I}$ -aFGF determined as described in Materials and Methods. Data were plotted as the mean and standard deviation of specifically bound  $^{125}\text{I}$ -aFGF as a function of the log of the total molar concentration of unlabeled FGF. Chick FGF from day-7 embryos was partially purified through heparin-agarose affinity columns as previously described (Seed et al., 1988). The protein concentration for chick FGF is reported as the log of the total molar FGF concentration.

#### Levels of FGF Receptor in Chick Embryos

Embryonic chick membranes were prepared and analyzed for FGF receptor levels by determination of specifically bound  $^{125}\text{I}$ -aFGF. From day 2, the earliest stage measured, to day 7, specific binding of  $^{125}\text{I}$ -aFGF remained relatively constant (Fig. 3 *a*), while in day-7–13 whole embryos, specific binding of  $^{125}\text{I}$ -aFGF decreased fourfold. In entire limbs and skeletal muscle tissue, FGF receptors exhibited a progressive decline from day 7 to 22 (1 d after hatching), when receptors were undetectable (Fig. 3 *b*). FGF receptors in heart tissue were also undetectable by day 19, whereas low levels of FGF receptor were still detectable in brain, eye, and liver (Fig. 3 *a*).

Putative FGF receptor polypeptides were identified by cross-linking  $^{125}\text{I}$ -aFGF to embryonic membrane preparations. Separation of the cross-linked complexes by SDS-PAGE and subsequent autoradiographic analysis distinguished two  $^{125}\text{I}$ -aFGF-FGF receptor complexes migrating at  $\sim 170$  and 215 kD (Fig. 4). The complex migrating at 170 kD is similar to migration of  $^{125}\text{I}$ -aFGF-FGF receptor complexes from mammalian cells in culture (Olwin and Hauschka, 1986, 1988, 1989). Assuming stoichiometric binding, subtraction of the molecular mass for aFGF from the two complexes yielded values of  $\sim 200$  and 150 kD for the FGF receptors. Whether the larger receptor, which has not been previously characterized, is distinct from or related to the smaller receptor is currently unknown. In agreement with the binding data presented in Fig. 3, the amounts of both cross-linked complexes appear relatively constant from day 2 to 7 and begin to decline between days 7 and 9 (Fig. 4). Disappearance of the 200-kD receptor appears to begin before the decline of the 150-kD receptor, suggesting these may



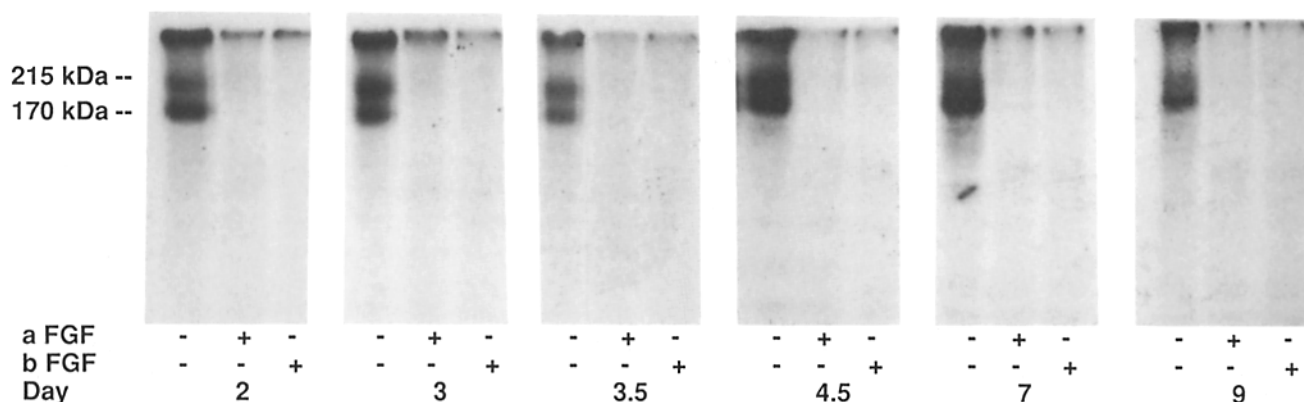
**Figure 3.** FGF receptor levels in embryonic chick. (a) FGF receptor levels in whole chick embryo from day 2 to 13 and in brain, eye, heart, and liver from day 17 and 19 embryonic membranes. (b) FGF receptor levels in limbs (L) from day 6 to 9 and in skeletal muscle (skm) from day 13 to 22 (1 day after hatching). FGF receptor levels were determined by specific binding of  $^{125}\text{I}$ -aFGF to staged embryos. Membrane assays contained 250 pM  $^{125}\text{I}$ -aFGF (saturating)  $\pm$  a 100-fold molar excess of unlabeled aFGF. Specific binding of  $^{125}\text{I}$ -aFGF reported as femtomoles  $^{125}\text{I}$ -aFGF bound per milligram membrane protein is equivalent to femtomoles FGF receptor per milligram protein, assuming stoichiometric interaction of  $^{125}\text{I}$ -aFGF with FGF receptor. Numbers in parentheses refer to the developmental stage as determined according to Hamburger and Hamilton (1951). For each data point at least two independent extracts were analyzed in two separate assays, each performed in triplicate, except for day-6 limb, where one extract was analyzed. For day-2-7 embryos, 20  $\mu\text{g}$  of protein was present per assay. For older embryos, assays were performed with 20  $\mu\text{g}$  and, when less than 10 fmol/mg bound  $^{125}\text{I}$ -aFGF was detected, 50  $\mu\text{g}$  of membrane protein. Data are plotted as the mean and standard deviation of these values.

be distinct, independently regulated FGF receptors. Both receptor complexes appear to bind aFGF and bFGF since a 100-fold molar excess of either growth factor competes for  $^{125}\text{I}$ -aFGF binding (Fig. 4).

#### Comparison of FGF Affinities in Early with Late Embryos

The binding and cross-linking data suggest that FGF receptor levels are regulated during chick embryogenesis. However, these experiments do not address whether changes in

the number of FGF receptors or changes in FGF receptor affinity are responsible for the apparent loss of  $^{125}\text{I}$ -aFGF binding and cross-linking. Therefore, the affinity and the number of  $^{125}\text{I}$ -aFGF binding sites were determined in membranes from day-3.5 whole embryos and day-19 brain. Scatchard analysis of equilibrium binding data revealed moderate changes in the  $K_d$  for  $^{125}\text{I}$ -aFGF binding to whole day-3.5 chick embryo membranes (70 pM) vs. day-7 (ranges from 100 to 232 pM) and day-19 embryonic brain ( $\sim$ 140 pM). Comparisons of  $K_d$ s between early and late embryonic stages are problematic due to the drastic overall loss



**Figure 4.** Cross-linking of  $^{125}\text{I}$ -aFGF to staged chick membranes. Crude membrane preparations of (50  $\mu\text{g}$ ) were incubated with 250 pM  $^{125}\text{I}$ -aFGF in the absence or in the presence of a 100-fold molar excess of unlabeled aFGF or bFGF. The membranes were washed, cross-linked with disuccinimidyl suberate, and processed for autoradiography as described in Materials and Methods. Identical amounts of membrane protein (25  $\mu\text{g}$ ) were loaded in each lane. Determinations of relative molecular weights were made from  $R_f$  values calculated using Bio-Rad Laboratories (Richmond, CA) high molecular weight SDS-PAGE standards (not shown). These data are representative of two independent experiments.

of FGF receptors: 100 fmol/mg for day-3.5 embryos vs.  $\leq 10$  fmol/mg for day-19 brains (the highest major tissue assayed at this stage). Such comparisons do, however, suggest that major changes in FGF receptor  $K_d$ s do not occur between these stages of development.

### Analysis of "Cryptic" FGF Receptors

Apparent modulation of FGF receptor levels could also occur due to differences in endogenous FGF levels between late and early embryos. Higher concentrations of FGF might lead to residual FGF during preparations of crude membrane extracts and consequently mask FGF binding sites. To assess this possibility, membrane preparations were exposed to an acid treatment that previous studies in our lab had shown releases bound  $^{125}\text{I}$ -aFGF from intact skeletal muscle (MM14) and Swiss 3T3 cells (Olwin, B. B., unpublished data). Such treatment was therefore expected to release bound endogenous chick FGF from membrane preparations. Embryonic (day 7) membranes were prepared as described in Materials and Methods, resuspended in neutral or acidic (pH 4.0) buffers for 30 min, centrifuged twice at 100,000 *g*, resuspended, and tested for FGF binding. Treatment of membranes with acidic buffers modestly increased the number of binding sites when compared with control membranes (Table I). To further substantiate that endogenous FGF was not released during the acidification procedure, initial supernatants from neutral and acid extraction were also adjusted to neutral pH and assayed for the presence of FGF by the same competitive binding procedure described in Fig. 2. No competition by supernatants from either neutral or acidified membranes was observed (our unpublished data); this assay has a detection limit for unbound FGF of 3 fmol or 50 pg (Seed et al., 1988).

To ensure that a large number of  $^{125}\text{I}$ -aFGF binding sites were not masked in day-19 embryos, brain and skeletal muscle membranes were treated with acidic buffers as described above. The number of  $^{125}\text{I}$ -aFGF binding sites in acid-treated brain membranes was approximately two-fold greater

than in control brain membranes when analyzed by Scatchard analysis of equilibrium binding data, similar to the changes observed for day-7 membranes (Table I). No specific binding for  $^{125}\text{I}$ -aFGF was observed in skeletal muscle membranes that were acidified or untreated (Table I). Consistent with results from day-7 embryos, acid treatment of day-19 embryos does not reveal a large number of binding sites masked by endogenous FGF. It thus appears that the loss of FGF binding sites observed during chick embryogenesis represents an actual loss of FGF receptor.

### Discussion

Chick embryonic membranes exhibit high affinity, specific binding to bovine  $^{125}\text{I}$ -aFGF. Because bovine aFGF and bFGF and chick FGF compete for the  $^{125}\text{I}$ -aFGF binding sites, all may bind common receptor polypeptides. Cross-linking  $^{125}\text{I}$ -aFGF to embryonic membranes identified two putative FGF receptors, one of which appears similar in biochemical characteristics to previously identified FGF receptors present in cultured mammalian cells ( $\sim 165$  kD; Olwin and Hauschka, 1989). A second FGF receptor of  $\sim 200$  kD, which has not been observed in mammalian cells, was detected by cross-linking to  $^{125}\text{I}$ -aFGF. Although neither the 150- nor 200-kD FGF-binding proteins were conclusively shown to be cell surface FGF receptors in this study, the 150-kD FGF-binding protein has been purified (Burrus and Olwin, 1989). Since the 150-kD FGF-binding protein binds  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF with high affinity and is a membrane glycoprotein present on chick embryo fibroblasts (Burrus and Olwin, 1989; Lueddecke, B. A., A. J. Kudla, and B. B. Olwin, manuscript in preparation), it most likely is an FGF receptor. Whether the 200-kD protein is a receptor for FGF or an FGF-binding protein incapable of FGF-mediated signal transduction is not known. However, its properties suggest it may be an FGF receptor, and thus the 200-kD FGF-binding protein is tentatively identified as an FGF receptor. Whether these receptors are distinct proteins or whether one form is derived from the other is currently unknown. The apparent differential changes in levels of the two receptors during embryogenesis suggest they may represent independent polypeptides (see below).

During chick development,  $^{125}\text{I}$ -aFGF binding is initially high and then decreases substantially. In the whole embryo, from day 2.5 to 7, the level of  $^{125}\text{I}$ -aFGF binding remains relatively constant. Specific binding first begins to decline around day 7 and is reduced 7- to 10-fold by day 13 (Figs. 3 and 4). A survey of several tissues in which a biological role for FGF has been proposed revealed that day-19 embryos have low levels of FGF receptor in brain, liver, and eye and no detectable receptors in skeletal muscle or heart tissue (Fig. 3). The loss of the 200-kD receptor, which is nearly complete by day 9, occurs before the decline of the 150-kD receptor, which is still present at  $\sim 50\%$  of its original level at day 9 (Fig. 4).

A decrease in FGF receptor numbers appears to be the primary cause for loss of  $^{125}\text{I}$ -aFGF binding sites since substantial numbers of cryptic receptors retaining bound endogenous FGF were not detected after acid treatment of membrane preparations. Membrane acidification results in 1.4- to 2.0-fold increases in the number of high affinity binding sites that is similar in samples with high (day 7) or low

Table I.  $^{125}\text{I}$ -aFGF Binding to Treated and Untreated Membranes

Age	Treatment*	$K_d$	Membrane protein bound
<i>d</i>		<i>pM</i>	<i>fmol/mg</i>
7	Control	232	90
	pH 4.0	269	140
19 (brain)	Control	$\sim 140^\ddagger$	$\sim 5^\ddagger$
	pH 4.0	160	10
19 (skeletal muscle)	Control	na $^\S$	$0.03 \pm 0.06^\parallel$
	pH 4.0	na $^\S$	0

\* Crude membranes prepared as described in Materials and Methods were treated with an acidic buffer (pH 4.0) to release bound endogenous FGF as described in the text.

$^\ddagger$  The low levels of  $^{125}\text{I}$ -aFGF binding did not allow an accurate determination of the  $K_d$  or numbers of binding sites. The data given represent approximate values.

$^\S$  na, not applicable. Saturation binding of  $^{125}\text{I}$ -aFGF was determined by incubating membranes (50  $\mu\text{g}$ ) with 200 pM  $^{125}\text{I}$ -aFGF in the absence and presence of excess unlabeled FGF as described in Materials and Methods. Reported values represent three independent determinations.

$^\parallel$  Note that the standard deviation for this measurement is greater than the value reported and, thus, these data are interpreted as indicating no specific binding of  $^{125}\text{I}$ -aFGF to skeletal muscle membranes.

(day 19) numbers of receptors, thus implying that the decrease in receptors is not an artifact of residual FGF (Table I). Further evidence in support of this conclusion is derived from equilibrium binding studies. The variation in  $K_d$  determined by Scatchard analyses of equilibrium binding data between different preparations of day-7 chick membranes using different  $^{125}\text{I}$ -aFGF preparations varies from two- to threefold, suggesting the differences in affinities between day 3.5, 7, and 13 are not significant. Within an individual experiment using a single preparation of membranes and  $^{125}\text{I}$ -aFGF, the variation in  $K_d$  was <1.5-fold. Assuming similar affinities of chick and bovine FGF, Scatchard analysis of equilibrium binding data from a range of developmental stages thus demonstrates insignificant changes in affinity of FGF receptor for  $^{125}\text{I}$ -aFGF but substantial decreases in FGF receptor number at older stages. Data from cross-linking experiments demonstrating a loss of FGF receptor polypeptides after day 7 are in agreement with these results.

Regulation of FGF receptor levels occurs during differentiation of skeletal muscle cells (Olwin and Hauschka, 1988) and in murine embryonal carcinoma cell lines (Rizzino et al., 1988). Differentiation of skeletal muscle myoblasts, which require FGF for growth and repression of differentiation, results in a permanent loss of FGF receptor with a time course that parallels that of differentiation (Olwin and Hauschka, 1988). Similar losses of FGF receptor occur in primary chick skeletal muscle cultures (Olwin, B. B., unpublished data). If muscle differentiation *in vivo* is accompanied by FGF receptor loss, then day-9 and older embryonic skeletal muscle tissue should exhibit progressively reduced levels of FGF receptor as increasing proportions of myoblasts become terminally differentiated. Since skeletal muscle precursor cells account for most of the tissue mass in day-4.5-7 limbs and since FGF receptors are known to be present in primary chick myoblasts *in vitro* (Seed and Hauschka, 1988; Olwin, B. B., unpublished data), the limb bud and also skeletal muscle data (Fig. 3 b) are consistent with the hypothesis that FGF receptors also disappear as myoblasts differentiate *in vivo*. These results provide further evidence in support of a model, previously proposed for cultured skeletal muscle cells, in which expression of FGF receptor levels may function to control temporal FGF-dependent processes (Lim and Hauschka, 1984; Olwin and Hauschka, 1988). Regulation of FGF receptor levels within embryonic limb myogenic tissue is particularly intriguing since strong immunopositive staining for FGF also occurs in the same myogenic regions (Joseph-Silverstein et al., 1989) and then, in adult muscle, becomes localized within the muscle basement lamina (DiMario et al., 1989).

Changes in FGF receptor levels during cellular differentiation in culture and during chick embryogenesis suggest that regulation of FGF receptor expression may play a key role in mediating the appropriate temporal and spatial actions of FGF during development. FGF-mediated developmental events occur as early as the gastrula stage in amphibian embryos (Slack et al., 1987; Kimelman and Kirschner, 1987). FGF and transforming growth factor  $\beta$ , which potentiates the inductive effect of FGF (Weeks and Melton, 1987; Rosa et al., 1988), are stored in unfertilized amphibian eggs as mRNAs (Weeks and Melton, 1987; Kimelman et al., 1988) and in unfertilized chick eggs as FGF protein (Seed et al., 1988). It seems likely that the mechanisms regulating meso-

derm induction will entail not only the controlled temporal and spatial release of FGF from the inducing endoderm cells but also the regulated expression of FGF receptors in responding cells.

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