IGF-I, insulin and FGFs induce outgrowth of the limb buds of amelic mutant chick embryos

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SUMMARY

IGF-I, insulin, FGF-2 and FGF-4 have been implicated in the reciprocal interactions between the apical ectodermal ridge (AER) and underlying mesoderm required for outgrowth and patterning of the developing limb. To study further the roles of these growth factors in limb outgrowth, we have examined their effects on the in vitro morphogenesis of limb buds of the amelic mutant chick embryos wingless (wl) and limbless (ll). Limb buds of wl and ll mutant embryos form at the proper time in development, but fail to undergo further outgrowth and subsequently degenerate. Wl and ll limb buds lack thickened AERs capable of promoting limb outgrowth, and their thin apical ectoderms fail to express the homeobox-containing gene Msx-2, which is highly expressed by normal AERs and has been implicated in regulating AER activity. Here we report that exogenous IGF-I and insulin, and, to a lesser extent, FGF-2 and FGF-4 induce the proliferation and directed outgrowth of explanted wl and ll mutant limb buds, which in vitro, like in vivo, normally fail to undergo outgrowth and degenerate. IGF-I and insulin, but not FGFs, also cause the thin apical ectoderms of wl and ll limb buds to thicken and form structures that grossly resemble normal AERs and, moreover, induce high level expression of Msx-2 in these thickened AER-like structures. Neither IGF-I, insulin nor FGFs induce expression of the homeobox-containing gene Msx-1 in the subapical mesoderm of wl or ll limb buds, although FGFs, but not IGF-I or insulin, maintain Msx-1 expression in normal (non-mutant) limb bud explants lacking an AER. The implications of these results to the relationships among the wl and ll genes, IGF-I/insulin, FGFs, Msx-2 and Msx-1 in the regulation of limb outgrowth is discussed.

Key words: IGF-I, insulin, FGF-2, FGF-4, wingless, limbless, limb outgrowth, apical ectodermal ridge (AER), Msx-2, Msx-1

INTRODUCTION

The outgrowth and patterning of the developing limb bud is dependent on reciprocal interactions between a thickened cap of epithelium at its distal apex called the apical ectodermal ridge (AER) and the underlying mesoderm of the limb bud (Saunders, 1977; Zwilling, 1961). The AER promotes the proliferation and outgrowth of the subridge mesoderm of the limb bud (Saunders, 1948), while suppressing its differentiation (Kosher et al., 1979; Solursh et al., 1981). The AER also maintains the activity of the zone of polarizing activity at the proximal posterior margin of the limb bud (Vogel and Tickle, 1993), which, in turn, appears to be the source of a morphogen, likely the product of the sonic hedgehog gene (Riddle et al., 1993), that regulates pattern formation along the anteroposterior axis of the limb bud (Saunders and Gasseling, 1968). The posterior subridge mesoderm of the limb bud is also the source of a factor or activity that maintains the thickness and outgrowth-promoting activity of the AER (Zwilling, 1961; Saunders and Gasseling, 1968).

Peptide growth factors including IGF-I, insulin and members of the FGF family have been implicated in the AER/subridge mesoderm interactions required for limb outgrowth and patterning. IGF-I and its receptor are expressed by the subbridge mesodermal cells of the limb bud growing out in response to the AER (Streck et al., 1992; Ralphs et al., 1990; Geduspan et al., 1992; Dealy and Kosher, 1995), and the maintenance of IGF-I expression by the subridge mesoderm is dependent on the AER (Dealy and Kosher, 1995). IGF-I, its receptor and IGF-binding proteins, which can modulate the activity of IGF-I, are also present in the AER itself (Ralphs et al., 1990; Geduspan et al., 1993; Streck et al., 1992; Green et al., 1994). Specific insulin receptors are also present in the chick limb bud during its outgrowth, although their spatial distribution has not yet been determined (Bassas et al., 1988). Exogenous IGF-I and insulin promote the proliferation and outgrowth of the subridge mesoderm of explanted chick limb buds lacking an AER, maintain the thickness and activity of the AER, and induce the formation of AER-like structures from the normally thin distal anterior ectoderm of the limb bud, while promoting dramatic outgrowth of the anterior mesoderm which normally undergoes little outgrowth (Dealy and Kosher, 1995). These observations have led to the hypothesis that IGF-I and insulin promote the outgrowth of limb mesoderm in response to the AER, and also regulate and/or maintain AER activity (Dealy and Kosher, 1995). Members of the FGF family, including FGF-4, FGF-2 and FGF-8, are expressed by the AER (Suzuki et al., 1992; Niswander and Martin, 1992; Savage et al., 1993; Ohuchi et al.,
To explore further the possible roles of IGF-I, insulin and FGFs in limb outgrowth, we have examined the effects of these growth factors on the in vitro morphogenesis of limb buds of the amelic mutant chick embryos wingless (w1) (Zwilling, 1961; Carrington and Fallon, 1984) and limbless (l1) (Carrington and Fallon, 1988). W1 and l1 are autosomal recessive mutations that directly affect the ectoderm, not the mesoderm, of limb buds (Carrington and Fallon, 1984, 1988). Limb buds of W1 and l1 mutant embryos form at the proper time in development, but fail to undergo further outgrowth and subsequently degenerate (Zwilling, 1961; Carrington and Fallon, 1984, 1988). W1 and l1 limb buds lack morphologically distinct and functional AERs capable of directing limb outgrowth (Carrington and Fallon, 1984, 1988), and the mesoderm of W1 limb buds appears to lack an activity required to maintain the thickness and activity of the AER (Zwilling, 1961). Here we report that exogenous IGF-I and insulin, and, to a lesser extent, FGF-2 and FGF-4, induce the proliferation and directed outgrowth of explanted W1 and l1 mutant limb buds. Furthermore, IGF-I and insulin, but not FGFs, induce the thin apical ectoderm of the mutant limb buds to form thickened AER-like structures that express high levels of Msx-2, a homeobox-containing gene that is characteristically expressed by thickened functional AERs and whose expression is absent in the apical ectoderm of W1 and l1 mutant limb buds. These results indicate that the W1 and l1 genes, IGF-I/insulin, Msx-2 and FGFs are involved in a regulatory network that controls the outgrowth and patterning of the developing limb.

MATERIALS AND METHODS

Windows were cut into eggs obtained from mating members of W1 and l1 heterozygous chickens maintained at the Storrs Agricultural Experiment Station, University of Connecticut, and both forelimbs of stage 20/21 (Hamburger and Hamilton, 1951) embryos were removed. Contralateral wing buds were cultured in the absence and presence of 10^{-8} M recombinant human IGF-I (Ciba-Geigy), 10^{-6} M or 10^{-9} M recombinant human insulin (Sigma), or 1 or 2\times10^{-8} M recombinant human FGF-2 or FGF-4 (Collaborative Research) and 100 ng/ml of heparan sulfate (Sigma) in individual wells of microtiter plates on nutrient agar substrates (Kosher et al., 1973) containing serum-free BGJb/F12 (7/3) medium supplemented with 0.1% bovine serum albumin (Dealy and Kosher, 1995). The donor embryos were reincubated until the phenotype of their hindlimb buds was clearly evident. In some experiments, the posterior 2/3 of the distal tip of normal stage 25 wing buds and normal stage 20/21 wing buds from which the AER or all limb ectoderm were removed as previously described (Dealy and Kosher, 1995) were cultured in the presence of IGF-I, insulin, FGF-2 or FGF-4.

The morphogenesis of the living explants was evaluated daily with a dissecting microscope and living explants were photographed. The relative sizes of various types of explants were determined by tracing the contours of 100x photographs of living explants onto acetate sheets, placing these upon graph paper and counting the number of graph units (0.5 cm^2) within the outlines of the explants.

Cell proliferation was examined as described (Dealy and Kosher, 1995) by immunohistochemical analysis of the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into nuclei engaged in DNA synthesis during a 1 hour labeling period with BrdU on day 2 of culture.

In situ hybridization was performed on serially sectioned explants using Msx-1- (Coelho et al., 1992a), Msx-2- (Coelho et al., 1991b) and IGF-I-specific (Dealy and Kosher, 1995) cDNA probes as previously described (Coelho et al., 1991b, 1992a; Dealy and Kosher, 1995).

RESULTS

Stage 20/21 W1 and l1 wing buds undergo little or no outgrowth when explanted to organ culture in serum-free medium (Fig. 1). On day 2 of culture, control W1 and l1 explants consist of small masses of tissue containing numerous large dark cells which are likely macrophages (Fig. 1) and are about the same size as at the time of their explantation (Table 1). The explants decrease in size on subsequent days (Table 1) suggesting they are degenerating. In contrast, in the presence of exogenous

<p>| Table 1. Relative sizes of control, IGF-I-, insulin-, FGF-2- and FGF-4-treated W1 and l1 wing bud explants |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Explant type</th>
<th>W1</th>
<th>l1</th>
<th>W1</th>
<th>l1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative size*</td>
<td>Treated/</td>
<td>Relative size*</td>
<td>Treated/</td>
<td>Relative size*</td>
</tr>
<tr>
<td></td>
<td>±s.e.m. (n)</td>
<td>control</td>
<td>±s.e.m. (n)</td>
<td>control</td>
<td>±s.e.m. (n)</td>
</tr>
<tr>
<td>1</td>
<td>control</td>
<td>19.3±1.4 (34)</td>
<td>–</td>
<td>9.4±1.9 (6)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10 nM IGF-I</td>
<td>50.9±3.4 (7)</td>
<td>2.6</td>
<td>66.5±2.1 (2)</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>1 μM insulin</td>
<td>47.5±4.8 (15)</td>
<td>2.5</td>
<td>63.3±1.7 (3)</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>1 nM insulin</td>
<td>32.6±2.4 (16)</td>
<td>1.7</td>
<td>43.8±9.5 (2)</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td>control</td>
<td>15.6±1.1 (47)</td>
<td>–</td>
<td>N.D.</td>
<td>–</td>
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<tr>
<td></td>
<td>20 nM FGF-2</td>
<td>38.2±2.0 (23)</td>
<td>2.4</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>20 nM FGF-4</td>
<td>33.8±2.2 (10)</td>
<td>2.2</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10 nM IGF-I</td>
<td>51.0±4.3 (5)</td>
<td>3.3</td>
<td>N.D.</td>
<td>–</td>
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</table>

*See Materials and Methods. The relative sizes of W1 and l1 wing buds at the time of explantation are 23.1±1.1 (n,7) and 22.0±1.3 (n,10), respectively. Groups 1 and 2 should be considered separately, since different batches of mutant eggs were used in each group and the response of mutant limb bud explants to exogenous growth factors differs somewhat among different batches of mutant eggs generated by different mating groups at different times of the year. In group 2, the IGF-treated W1 explants are significantly larger than W1 explants treated with FGF-2 (P=0.0131) or FGF-4 (P=0.0015) as determined by paired t-tests; and, the IGF-I-treated l1 explants are significantly larger than l1 explants treated with FGF-2 (P=0.0224) or FGF-4 (P=0.0022). N.D.=not determined.
IGF-I and insulin, the mesoderm of contralateral *wl* and *ll* wing buds undergoes considerable outgrowth (Fig. 1). 10^{-8} M IGF-I- and 10^{-6} M insulin-treated *wl* and *ll* wing bud explants undergo outgrowth to about the same extent, being about 2- to 3-fold larger than control explants on day 2 and about 4- to 7-fold larger on day 6 (Table 1). Exogenous FGF-2 and FGF-4 (1 or 2\times10^{-8} M) also induce the directed outgrowth of explanted *wl* and *ll* mutant wing buds by promoting the proliferation of cells in the subapical mesoderm of the explants (Fig. 2). Immunohistochemical analysis of bromodeoxyuridine incorporation demonstrates that a highly proliferating population of cells, 30-45% of which are engaged in DNA synthesis, are present in the mesoderm underlying the apical ectoderm of day 2 IGF-I-, insulin- and FGF-treated *wl* and *ll* wing bud explants (Fig. 2). A similar population of highly proliferating cells (about 40% engaged in DNA synthesis) are present in the mesoderm subjacent to the AER of normal (non-mutant) wing bud explants. However, DNA synthetic activity in the subapical mesoderm of IGF-I-, insulin- and FGF-treated *wl* and *ll* explants is comparable in thickness to the AER of normal (non-mutant) wing bud explants (Fig. 3A-G). The thin nonfunctional apical ectoderm of stage 20/21 *ll* and *wl* mutant limb buds exhibits no detectable IGF-I mRNA expression (data not shown). In addition, the thin apical ectoderm of stage 20/21 *ll* (Coelho et al., 1991a; Robert et al., 1991) and *wl* (data not shown) limb buds exhibits virtually no expression of the homeobox-containing gene *Mx*-2, a gene that is characteristically highly expressed by thickened functional AERs (Coelho et al., 1991b, 1992b). The ectoderm of control (non-treated) *wl* and *ll* explants continues to exhibit no detectable *Mx*-2 expression (Fig. 3H, K). However, the thickened apical ectoderm that forms in contralateral IGF-I-
and insulin-treated *wl* and *ll* wing bud explants exhibit a high level expression of *Msx-2* (Fig. 3L,L) comparable to that observed in the AER of normal (non-mutant) wing bud explants (Fig. 3N). In contrast to IGF-I and insulin, FGF-2 and FGF-4 do not induce high level expression of *Msx-2* in the apical ectoderms of *wl* and *ll* mutant limb bud explants (Fig. 3J,M). Thus, IGF-I and insulin, but not FGF-2 or FGF-4, induce high level expression of the AER-characteristic homeobox-containing gene *Msx-2* in the apical ectoderm of explanted *wl* and *ll* mutant limb buds, while causing the ectoderm to thicken and form a structure that resembles an AER.
The homeobox-containing gene *Msx-1* is expressed at high levels in the subridge mesoderm of stage 20/21 normal (non-mutant) limb buds (Coelho et al., 1991a,b) and normal limb bud explants (Fig. 4A). In contrast, little or no *Msx-1* is expressed in the subapical mesoderm of stage 20/21 *ll* (Coelho et al., 1991a; Robert et al., 1991) or *wl* (data not shown) limb buds or in mutant limb bud explants (Fig. 4B,E); and, neither IGF-I, insulin, FGF-2 nor FGF-4 induce high level *Msx-1* expression in the mesoderm of mutant limb bud explants (Fig. 4C-G).

Although FGF-2 and FGF-4 at the concentrations examined do not induce *Msx-1* expression in the subapical mesoderm of *wl* or *ll* limb buds, they do maintain high level *Msx-1* expression in the subridge mesoderm of normal (non-mutant) limb bud explants cultured in the absence of the AER (Fig. 5) or all limb ectoderm (data not shown). Control explants of the posterior distal tip of stage 25 normal wing buds (Fig. 5) or stage 20/21 normal wing buds (data not shown) lacking an AER (Fig. 5) or all limb ectoderm (data not shown) fail to undergo outgrowth and cease expressing *Msx-1*, whereas the subridge mesoderm of FGF-treated explants of these types undergoes considerable outgrowth and the outgrowing cells exhibit high level expression of *Msx-1* (Fig. 5). In contrast, IGF-I and insulin fail to maintain high level *Msx-1* expression in the mesoderm of normal (non-mutant) limb buds lacking an AER (Fig. 5) or limb ectoderm (data not shown), although these growth factors promote dramatic outgrowth of the subridge mesoderm in such explants (Fig. 5 and Dealy and Kosher, 1995).

**DISCUSSION**

IGF-I is expressed by the subridge mesodermal cells of the limb bud growing out in response to the AER as well as by the AER itself (Ralphs et al., 1990; Geduspan et al., 1992, 1993; Streck et al., 1992; Dealy and Kosher, 1995) and specific insulin receptors are present in the developing limb during its outgrowth (Bassas et al., 1988). Our finding that IGF-I and insulin promote the proliferation and directed outgrowth of explanted *wl* and *ll* mutant limb buds which normally fail to undergo outgrowth and degenerate provides strong support for the hypothesis that these growth factors are involved in the reciprocal interactions between the AER and subridge mesoderm required for limb outgrowth (Dealy and Kosher, 1995). The present studies do not allow us to determine if the outgrowth of the mutant limb buds in response to IGF-I and insulin results from a direct effect on the mesoderm, the apical ectoderm or both of these tissues. Several observations, however, indicate that IGF-I and insulin can affect both the subridge mesoderm and AER of the developing limb bud. The maintenance of IGF-I expression by the subridge mesoderm is dependent on the AER (Dealy and Kosher, 1995). Exogenous IGF-I and insulin promote the outgrowth of the subridge mesoderm of explanted limb buds lacking an AER indicating that endogenous IGF-I and/or insulin present in the subridge mesoderm during normal development may promote its proliferation and outgrowth in response to the AER (Dealy and Kosher, 1995). The results of the present and previous studies indicate that IGF-I and insulin can also affect the activity of the AER. We have previously found that these growth factors induce the formation of thickened AER-like structures that express high levels of the AER-characteristic homeobox-containing gene *Msx-2* from the normally thin distal anterior ectoderm of the chick limb bud (Dealy and Kosher, 1995). Here we have found that IGF-I and insulin induce the normally thin nonfunctional distal apical ectoderm of *wl* and *ll* mutant limb buds to thicken and form a structure that grossly resembles an AER, and induce high level expression of *Msx-2* in these thickened AER-like structures. Taken together, these results are consistent with our previous suggestion (Dealy and Kosher, 1995) that endogenous IGF-I and insulin may promote the proliferation and outgrowth of the subridge mesoderm in response to the AER and may also be involved in regulating and/or maintaining AER activity.

It is of particular interest that IGF-I and insulin induce the expression of *Msx-2* in the apical ectoderm of *wl* and *ll* mutant limb buds. IGF-I and *Msx-2* are not expressed in detectable amounts in the apical ectoderm of *wl* and *ll* mutant limb buds,
indicating their expression in the AER is directly or indirectly regulated by the products of the \(wl\) and \(ll\) genes. The fact that IGF-I and insulin induce \(Msx-2\) expression in \(wl\) and \(ll\) apical ectoderms suggests that the products of the \(wl\) and \(ll\) genes may regulate the expression of IGF-I in the normal AER and that, in turn, IGF-I regulates \(Msx-2\) expression in the AER. Although the function of \(Msx-2\) in the AER is not known, it has been suggested that it may regulate, perhaps in conjunction with other regulatory genes such as members of the \(Dlx\) family (Ferrari et al., 1995), the expression of members of the FGF family, which then directly mediate the effects of the AER on the subridge mesoderm.

FGF-2 and FGF-4 also induce the directed outgrowth of explanted \(wl\) and \(ll\) mutant limb buds, although to a significantly lesser extent than IGF-I. Furthermore, in contrast to IGF-I and insulin, FGFs do not induce expression of \(Msx-2\) in the mutant apical ectoderms. These results suggest that FGFs promote the outgrowth of the mutant limb buds by acting on the mesoderm, not the ectoderm, whereas IGF-I and insulin may promote outgrowth by acting on both the mesoderm and apical ectoderm of the mutant limb buds (see above). The maintenance of IGF-I expression by the subridge mesoderm of normal limb buds is dependent on the AER (Dealy and Kosher, 1995). Since FGFs produced by the AER appear to mediate its outgrowth and patterning effects (Niswander and Martin, 1993; Niswander et al., 1993; Riley et al., 1993; Fallon et al., 1994; Taylor et al., 1994; Vogel and Tickle, 1993; Anderson et al., 1993; Laufer et al., 1994), one of their effects may be to maintain the expression of IGF-I by the subridge mesoderm, and these growth factors may then interact in concert to promote the proliferation and outgrowth of the subridge mesoderm. In several systems, FGFs regulate the expression of IGF-I and/or its receptor (Villaudy et al., 1991; Rosenthal et al., 1991; Drago et al., 1991) and, interestingly, the FGF-induced proliferation of cultured neuroepithelial cells is dependent on endogenous IGF-I (Drago et al., 1991).

On the basis of all of the above observations, we suggest the following model to serve as the basis for further experimentation (see Fig. 6). The products of the \(wl\) and \(ll\) genes directly or indirectly regulate and/or maintain the expression of IGF-I by the AER, and IGF-I, in turn, regulates the expression of the homeobox-containing gene \(Msx-2\) in the AER. \(Msx-2\), perhaps in conjunction with other regulatory genes such as members of the \(Dlx\) family (Ferrari et al., 1995), may then regulate the expression of FGFs, which mediate the effects of the AER on the mesoderm.
the subridge mesoderm. One of the effects of FGFs secreted by the AER may be to maintain IGF-I expression by the subridge mesoderm, which promotes the proliferation and outgrowth of the mesoderm.

**Mx** is a homeobox-containing gene that is highly expressed by the subridge mesoderm of normal limb buds (Coelho et al., 1992a). Maintenance of Mx expression by the subridge mesoderm is dependent on the AER, since its expression is severely impaired in the subapical mesoderm of stage 20/21 *ll* (Coelho et al., 1991a; Robert et al., 1991) and *wl* (the present study) mutant limb buds, which lack morphologically distinct and functional AERs, and surgical extirpation of the AER in vivo (Ros et al., 1992) or in vitro (the present study) results in a cessation of Mx expression in the subridge mesoderm. In the present study, we have found that neither IGF-I, insulin, FGF-2 nor FGF-4 induce high level expression of Mx in the subapical mesoderm of stage 20/21 *wl* or *ll* mutant limb bud explants, although each of these growth factors promotes the proliferation and outgrowth of the mutant limb buds. This indicates that Mx expression is not required for the outgrowth and proliferation of limb mesoderm in response to FGFs, IGF-I or insulin. Interestingly, however, FGF-2 and FGF-4 maintain high level expression of Mx in normal limb bud explants lacking an AER or all limb ectoderm, while promoting outgrowth of the subridge mesoderm. FGF-2-soaked beads also maintain Mx expression in the subridge mesoderm of limb buds in ovo following surgical extirpation of the AER (Fallon et al., 1994). In interpreting these results, it is important to note that, although the AER maintains Mx expression by the subridge mesoderm, it does not regulate the onset of Mx expression in this tissue, since Mx expression is relatively normal in the subridge mesoderm of *ll* mutant limb buds initially following their formation (Coelho et al., 1991a; Robert et al., 1991). These observations are consistent with the suggestion of Fallon et al. (1994) that the effect of the AER and FGFs secreted by it on the subridge mesoderm is permissive, not instructive. That is, regulatory genes and signaling molecules produced by the subridge mesoderm that control the proliferation and patterning of the mesoderm are already in place when the AER is induced during the initial formation of the limb bud and the AER, via FGFs secreted by it, simply maintains or stabilizes the inherent morphogenetic properties of the mesoderm.

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