(which correspond to atrial and ventricular depolarization, respectively) typical for mice. There was no evidence for cardiac arrhythmia in graft-bearing animals, despite the apparent presence of a high degree of intercellular coupling between grafted and host cardiomyocytes.

The donor transgenic cardiomyocytes used in this study were mitotically active when grafted. Although it is presently not clear if cell cycling is a prerequisite for successful graft formation, the time course of [^{3}H]thymidine incorporation and the use of donor transgenic cardiomyocytes from different stages of development should address this issue. If cardiomyocyte cycling is in fact required for graft formation, the ability to transiently induce proliferation in vitro could mean that biopsied adult cardiomyocytes could be used for autologous grafting experiments. Recent studies using transformed cardiomyocytes derived from transgenic mice have identified three endogenous proteins that form stable complexes with SV40 large T antigen (*13*). By analogy to skeletal muscle (*14*), these proteins may mediate cardiomyocyte terminal differentiation and consequently represent potential targets (either independently or in combination) with which to engender transient cardiomyocyte proliferation in vitro. In addition to fetal hearts, cardiomyocytes derived from embryonic stem cells (*15*) may be a viable source for donor cardiomyocytes. Intracardiac grafting of such cells might be useful for myocardial repair, provided that the grafted cells can contribute to myocardial function.

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5. The MHC promoter consisted of 4.5 kb of 5′ flanking sequence and 1 kb of the gene encompassing exons 1 through 3 up to but not including the initiation codon [J. Gulick, A. Subramaniam, J. Neumann, J. Robbins, *J. Biol. Chem.* **266**, 9180 (1991); A. Subramaniam *et al.*, *ibid.*, p. 24613]. The nLAC reporter was modified to carry both a eukaryotic translation initiation site and the SV40 nuclear localization signal [E. H. Mercer, G. W. Hoyle, R. P. Kapur, R. L. Brinster, R. D. Palmiter, *Neuron* **7**, 703 (1991)]. The transgene also carried an intron, as well as transcriptional termination and polyadenylation signals from the mouse protamine 1 gene. For generation of transgenic mice, MHC-nLAC insert DNA was purified by adsorption onto glass beads, dissolved at a concentration of 5 μg/ml, and microinjected into the nuclei of one-cell inbred C3Heb/FeJ embryos according to established protocols [B. Hogan, F. Costantini, E. Lacy, in *Manipulating the Mouse Embryo—A Laboratory Manual*, F. Constantini and E. Lacy, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)]. Analysis by polymerase chain reaction (PCR) was used to identify founder animals and to monitor transgene segregation. The sense strand primer 5′-GGTGGGGGCTCTTCAC-CCCCAGACCTCTCC-3′ was localized to the MHC promoter, and the antisense strand primer 5′-GCCAGGGTTTTCCCAGTCACGACGTTGT-3′ was localized to the nLAC reporter. PCR analyses were as described [M. E. Steinhelper, K. L. Cochrane, L. J. Field, *Hypertension* **16**, 301 (1990)].
6. Transgenic animals were heparinized (10,000 U/kg, intraperitoneally) before they were killed by cervical dislocation. Hearts were placed in a beaker of gassed (95% O_2, 5% CO_2) KHB buffer (105 mM NaCl, 20 mM $NaHCO_3$, 3.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM $CaCl_2$, 1 mM mannitol, 10 mM taurine, 10 mM dextrose, and 5 mM sodium pyruvate). Hearts were then hung by the aorta and perfused with gassed KHB (0.5 ml/min at 37°C) containing 2.5 mM EGTA for 5 min, followed by 0.17% collagenase (type I; Worthington Biochemical, Freehold, NJ) in KHB. Hearts were perfused until flaccid and the ventricles were minced with scissors, and isolated cells were obtained by trituration with a Pasteur pipette. After at least 1 hour of formalin fixation, suspensions were filtered and smeared onto positively charged slides (Superfrost Plus, Fisher, Pittsburgh, PA) and allowed to dry.
7. For isolation of single cells for injection, females with 15-day-old embryos (onset of pregnancy determined by vaginal plugs) were killed by cervical dislocation. Embryos were removed and decapitated, hearts were harvested under PBS, and ventricles and atria were separated. Transgenic ventricles (identified by cardiac β-gal activity) were digested in 0.1% collagenase (Worthington) in DPBS (Dulbecco's phosphate-buffered saline; Sigma) for 45 min and were triturated with a Pasteur pipette in PC-1 medium (Ventrex, Coons Rapids, MN) with 10% fetal bovine serum, resulting in a suspension of single cells.
8. Immediately after isolation, fetal cardiomyocytes were washed three times with DPBS and injected directly into the ventricular myocardium of syngeneic mice (Jackson Laboratory, Bar Harbor, ME) under open-heart surgery as described [H. A. Rockman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8277 (1991)]. Cells (1×10^4 to 10×10^4) were injected in a volume of 2 to 3 μl with the use of a plastic syringe fitted with a 30-gauge needle.
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Characterization of Type I Receptors for Transforming Growth Factor–β and Activin

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Transforming growth factor–β (TGF-β) and activin exert their effects by binding to heteromeric complexes of type I and type II receptors. The type II receptors for TGF-β and activin are transmembrane serine-threonine kinases; a series of related receptors, denoted activin receptor–like kinase (ALK) 1 to 5, have recently been identified, and ALK-6 is described here. ALK-5 has been shown to be a functional TGF-β type I receptor. A systematic analysis revealed that most ALKs formed heteromeric complexes with the type II receptors for TGF-β and activin after overexpression in COS cells; however, among the six ALKs, only ALK-5 was a functional TGF-β type I receptor for activation of plasminogen activator inhibitor–1, and only ALK-2 and ALK-4 bound activin with high affinity.

The TGF-β proteins belong to a family of dimeric proteins that regulate the growth, differentiation, and metabolism of many cell types (*1*), and they are members of a larger superfamily of structurally related proteins that includes activins, inhibins, bone morphogenetic proteins, and Müllerian inhibiting substance. Both TGF-βs and activins exert their effects through binding to specific cell surface receptors (*2*). The TGF-β type I (53 kD) and type II (75 kD) receptors are indispensable for signal transduction and form heteromeric complexes on ligand binding (*3*, *4*). The

type II receptors for TGF-β (TβR-II) (5) and activin (ActR-II) (6) are both transmembrane serine-threonine kinases.

A series of receptor-like serine-threonine kinases have been identified that were termed ALK-1 to -5 (*7–12*). The sizes of ALKs are similar to the reported sizes of the type I receptors for TGF-β and activin (*2, 6*). Moreover, ALK-2 (Tsk 7L) (*9*) and ALK-5 (*8*) form heteromeric complexes with TβR-II and bind TGF-β, and ALK-5 forms a functional receptor complex (*8, 13*). ALK-1 and ALK-2 have been reported to bind activin (*12, 14*). We identified a sixth member of the ALK family and systematically investigated which ALKs can act as type I receptors for TGF-β and activin.

We obtained the sixth clone, ALK-6, by screening a complementary DNA (cDNA) library from 12-day mouse embryos with a probe from a part of the kinase domain of ALK-4 under low stringency hybridization conditions (Fig. 1A). A typical hydrophobic leader sequence is not observed in the NH_2-terminus of the translated region; however, the ALK-6 protein was efficiently expressed at the cell surface. RNA blot analysis revealed a transcript of 7.5 kb in brain, and a weaker hybridization was seen with mRNA from the lung (Fig. 1B). A phylogenetic tree based on the similarities between the kinase domains of mammalian receptors with serine-threonine kinase activity (*5–8, 15*) is shown in Fig. 1C. The ALKs are more similar to each other than to the TGF-β and activin type II receptors.

Affinity cross-linking studies with ^{125}I-labeled TGF-β1 confirmed that COS-1 cells express low or undetectable amounts of TGF-β type I or type II receptors (*16*). Transfection of cDNAs for ALKs into COS-1 cells did not enhance binding of ^{125}I–TGF-β1 (*3, 4, 9*), apart from a very weak binding of ^{125}I–TGF-β1 to ALK-5 (*16*). When COS-1 cells were cotransfected with TβR-II and ALK cDNAs, analyzed by affinity cross-linking, and then immunoprecipitated with a specific antiserum to TβR-II, each of the ALKs bound ^{125}I–TGF-β1 and was coimmunoprecipitated with the TβR-II complex, but ALK-5 formed such complexes more efficiently than the other ALKs (Fig. 2A). The size of the cross-linked complex was larger for ALK-3 than for the other ALKs, consistent with its slightly larger size (*7*). When the cross-linked complexes were immunoprecipitated by antibodies specific for the different ALKs, each of the ALKs was immunoprecipitated in complex with TβR-II (Fig. 2B). ALK-5 formed a heteromeric complex with TβR-II more efficiently than did the other ALKs.

We investigated which ALKs serve as TGF-β type I receptors in nontransfected, TGF-β–responsive cell lines. Several different cell lines were incubated with ^{125}I–TGF-β1 and then cross-linked to the receptors. Proteins were immunoprecipitated with antisera to different ALKs. Only the antiserum to ALK-5 efficiently immunoprecipitated the cross-linked type I and type II receptor complexes in mink lung epithelial cells (Mv1Lu), porcine aortic endothelial (PAE) cells (Fig. 2C), and human foreskin fibroblasts (*16*), although ALK-2 and ALK-4 are also expressed in these cell lines and fibroblasts also express ALK-1 and ALK-3 (*16*).

We investigated whether ALKs restored responsiveness to TGF-β in the R mutant of Mv1Lu cells (clone 4-2) that lacks functional TGF-β type I receptors but has intact type II receptors (*3*). The R mutant cells were transfected with the cDNAs for ALKs or a control plasmid, and the production of plasminogen activator inhibitor (PAI)–1 was measured after the addition of TGF-β1. Wild-type Mv1Lu cells and the R mutant cells transfected with the ALK-5 cDNA responded to TGF-β1 (*8, 13*) and produced a characteristic 45-kD PAI-1 protein in the extracellular matrix (Fig. 2D). In contrast, the R mutant cells that were transfected with the other ALKs did not produce PAI-1 with the addition of TGF-β1. Thus, only ALK-5 efficiently formed a signaling TGF-β receptor complex with regard to PAI-1 induction.

We also investigated whether ALKs bind activin in the presence of ActR-II. COS-1 cells were cotransfected with the cDNAs for ALKs and ActR-II and affinity labeled with ^{125}I-labeled activin A. Cross-linked proteins

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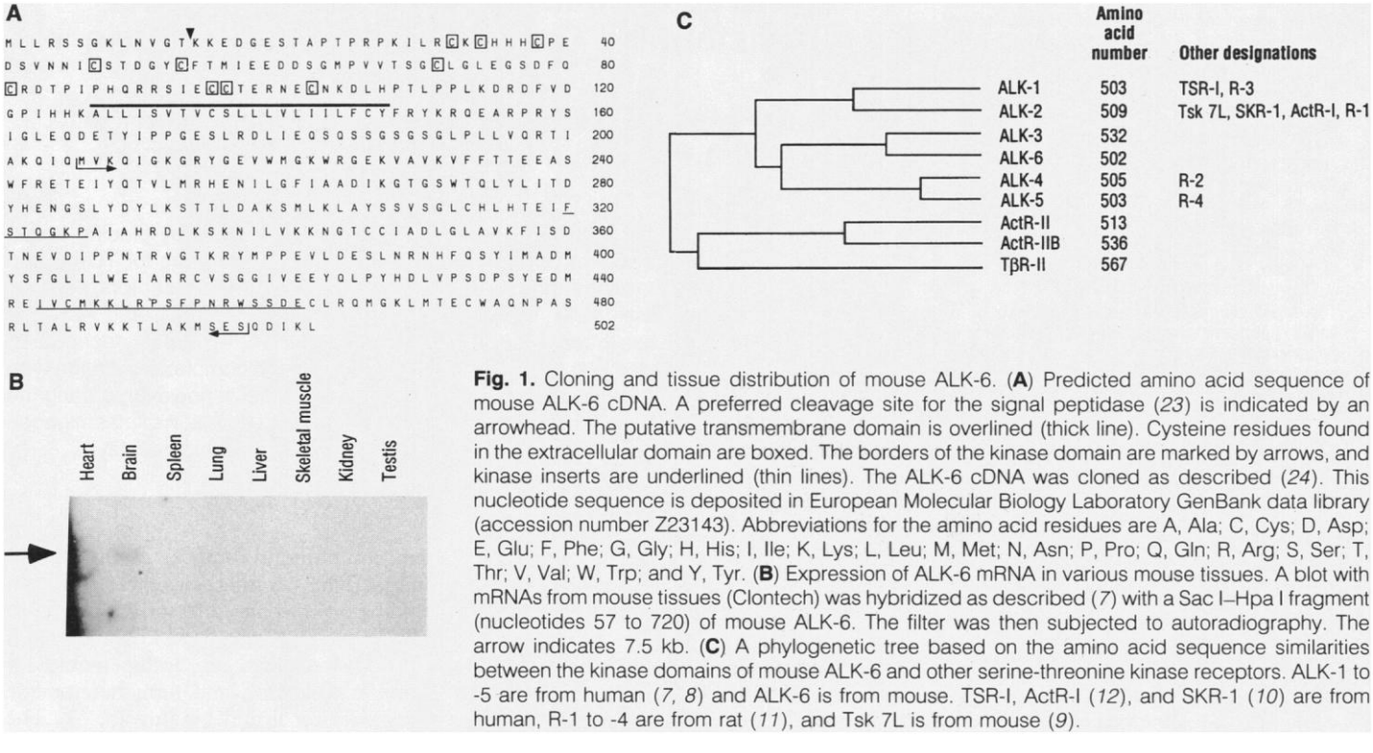

Fig. 1. Cloning and tissue distribution of mouse ALK-6. (**A**) Predicted amino acid sequence of mouse ALK-6 cDNA. A preferred cleavage site for the signal peptidase (*23*) is indicated by an arrowhead. The putative transmembrane domain is overlined (thick line). Cysteine residues found in the extracellular domain are boxed. The borders of the kinase domain are marked by arrows, and kinase inserts are underlined (thin lines). The ALK-6 cDNA was cloned as described (*24*). This nucleotide sequence is deposited in European Molecular Biology Laboratory GenBank data library (accession number Z23143). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Expression of ALK-6 mRNA in various mouse tissues. A blot with mRNAs from mouse tissues (Clontech) was hybridized as described (*7*) with a Sac I–Hpa I fragment (nucleotides 57 to 720) of mouse ALK-6. The filter was then subjected to autoradiography. The arrow indicates 7.5 kb. (**C**) A phylogenetic tree based on the amino acid sequence similarities between the kinase domains of mouse ALK-6 and other serine-threonine kinase receptors. ALK-1 to -5 are from human (*7, 8*) and ALK-6 is from mouse. TSR-I, ActR-I (*12*), and SKR-1 (*10*) are from human, R-1 to -4 are from rat (*11*), and Tsk 7L is from mouse (*9*).

were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). After overexpression in this system, all ALKs except for ALK-3 appeared to bind activin A in the presence of ActR-II (Fig. 3A). This could be more clearly demonstrated by affinity cross-linking and immunoprecipitation with antisera to ActR-II or ALKs. Both ALK-2 and ALK-4 bound ^{125}I-labeled activin A and were coimmunoprecipitated with ActR-II by the antiserum to ActR-II or ALKs (Fig. 3B). The ALK-1, ALK-5, and ALK-6 also bound ^{125}I-labeled activin A, but with lower efficiencies compared with ALK-2 and ALK-4. ALK-3 showed little binding of ^{125}I-labeled activin A.

To investigate which ALKs are physiological activin type I receptors, we attempted to identify endogenous activin type I receptors expressed in activin-responsive cells. The Mv1Lu cells and R mutant cells express both type I and type II receptors for activin and produce PAI-1 on the addition of activin A (*16*). The Mv1Lu cells were labeled with ^{125}I-labeled activin A. After cross-linking, the receptors were immunoprecipitated with antisera to ActR-II or ALKs. The type I and type II receptor complexes in Mv1Lu cells were immunoprecipitated only by the antisera to ALK-2, ALK-4, and ActR-II (Fig. 3C). Similar results were obtained in the R mutant cells (*16*). PAE cells do not bind activin because of the lack of type II receptors for activin; however, after transfection of a chimeric receptor containing the extracellular domain and the COOH-terminal tail of ActR-II and the kinase domain of TβR-II, the cells (PAE–Chim A) bound ^{125}I-labeled activin A and were growth-inhibited by the addition of activin A (*16*). Activin type I receptor complexes in PAE–Chim A cells were immunoprecipitated by the ALK-2 and ALK-4 antisera (Fig. 3C). These results suggest that both ALK-2 and ALK-4 act as physiological type I receptors for activin in these cells.

Because there are no known established cell lines that lack type I receptors or both type I and type II receptors for activin, we could not study the restoration of activin signals by the transfection of ALKs. In R mutant cells (clone R1B), ALK-2, in combination with ActR-II, was reported to transduce an activin-induced transcriptional response (*12*). However, we found that the R mutant (clone 4-2 and R1B) bound activin A and produced PAI-1 on stimulation with activin A without transfection of ALKs or ActR-II cDNA, and no change in the amount of PAI-1 production was observed after cotransfection of ActR-II and ALKs (*16*).

The type I receptors (ALKs) cannot bind ligand in absence of type II receptors and there exists no cross-binding between TGF-β and activin to the type II receptors (*6*, *15*). In contrast, binding of the ligands to type I receptors (ALKs) appears less strict, as many of them can bind TGF-β1 and activin A in the presence of the respective type II receptors when expressed in large amounts in COS-1 cells. However, binding studies with TGF-β– and activin-responsive Mv1Lu and PAE cells showed

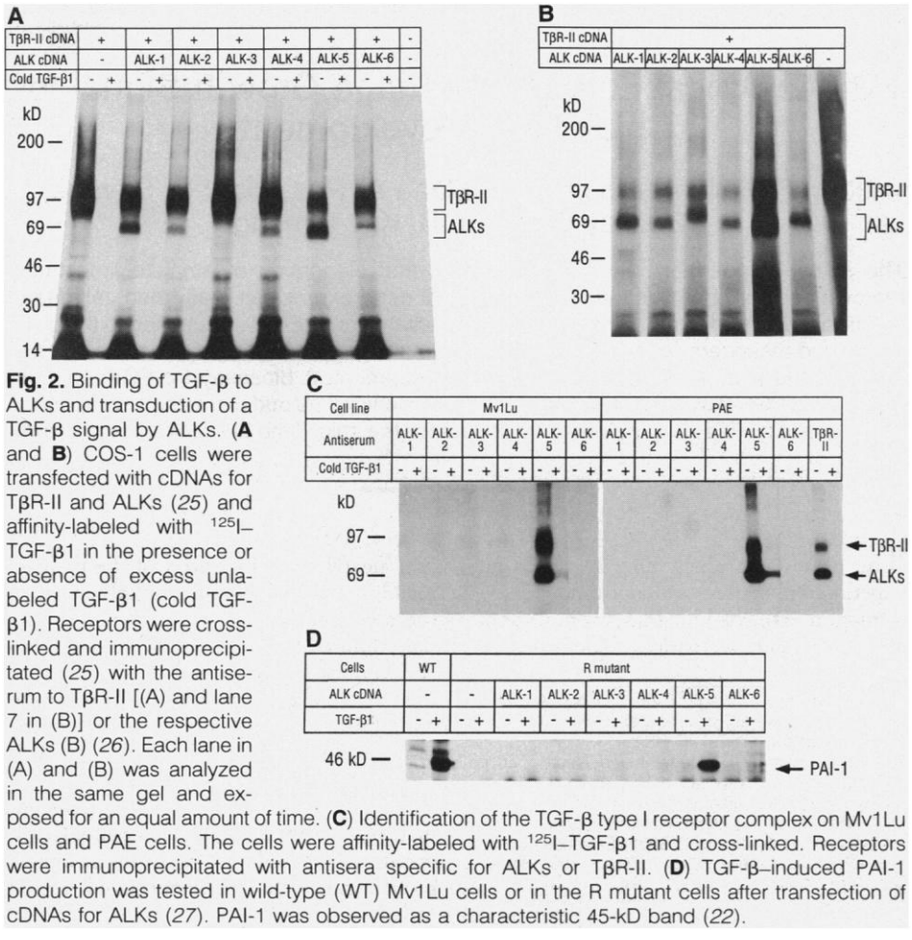

Fig. 2. Binding of TGF-β to ALKs and transduction of a TGF-β signal by ALKs. (**A** and **B**) COS-1 cells were transfected with cDNAs for TβR-II and ALKs (*25*) and affinity-labeled with ^{125}I–TGF-β1 in the presence or absence of excess unlabeled TGF-β1 (cold TGF-β1). Receptors were cross-linked and immunoprecipitated (*25*) with the antiserum to TβR-II [(A) and lane 7 in (B)] or the respective ALKs (B) (*26*). Each lane in (A) and (B) was analyzed in the same gel and exposed for an equal amount of time. (**C**) Identification of the TGF-β type I receptor complex on Mv1Lu cells and PAE cells. The cells were affinity-labeled with ^{125}I–TGF-β1 and cross-linked. Receptors were immunoprecipitated with antisera specific for ALKs or TβR-II. (**D**) TGF-β–induced PAI-1 production was tested in wild-type (WT) Mv1Lu cells or in the R mutant cells after transfection of cDNAs for ALKs (*27*). PAI-1 was observed as a characteristic 45-kD band (*22*).

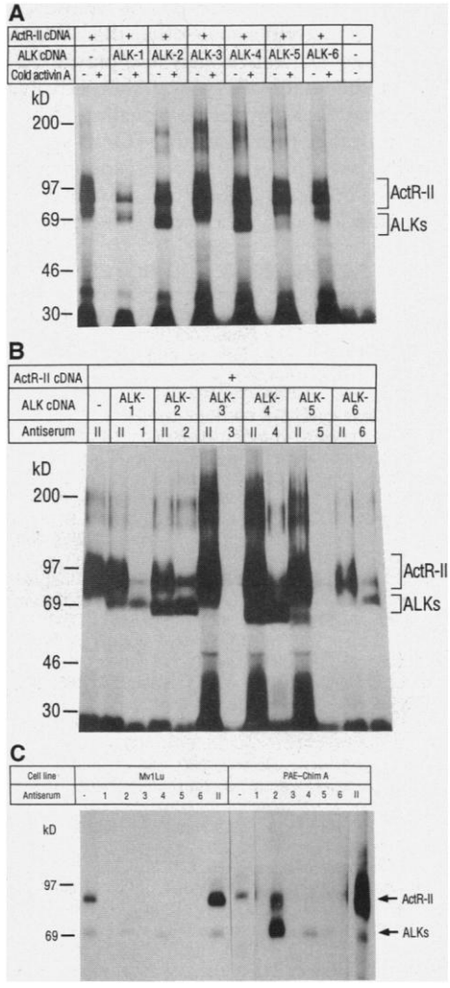

Fig. 3. Identification of activin type I receptors. (**A**) COS-1 cells were cotransfected with cDNAs for ALKs and ActR-II (*25*) and analyzed for binding and cross-linking of ^{125}I-labeled activin A in the presence or absence of excess unlabeled activin A (cold activin A) (*25*). (**B**) Binding and cross-linking of ^{125}I-labeled activin A to the transfected COS-1 cells and then immunoprecipitation with antisera to ActR-II (II) or ALKs (1 to 6) (*26*). (**C**) Binding and cross-linking of ^{125}I-labeled activin A to Mv1Lu cells and PAE–Chim A cells (*28*). Cell lysates were analyzed without (−) or with immunoprecipitation with antibodies specific for ALK-1 to -6.

that only antisera against ALK-5 immunoprecipitated cross-linked TGF-β type I receptor complexes, and antisera against ALK-2 and ALK-4 immunoprecipitated cross-linked activin type I receptor complexes. Moreover, among the six ALKs, only ALK-5 efficiently restored the TGF-β activation of PAI-1 synthesis in mutant Mv1Lu cells.

The low efficiency binding of TGF-β to ALKs other than ALK-5 and of activin to ALKs other than ALK-2 and ALK-4, when expressed together with type II receptors in COS-1 cells, appears not to represent true physiological interactions. The possibilities remain, however, that other ALKs may bind TGF-β2 or TGF-β3 or transduce TGF-β or activin signals other than PAI-1 induction. Differences may also prevail between different cell types. Alternatively, the other ALKs might be signaling receptors for other ligands in the TGF-β superfamily, such as bone morphogenetic proteins or Müllerian inhibiting substance, in combination with other type II receptors.

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25. We generated transient expression plasmids encoding ALK-1 through -6, TβR-II, and ActR-II by subcloning into the pSV7d expression vector (*18*) or into the pcDNA I expression vector (Invitrogen). For transient transfection, COS-1 cells (American Type Culture Collection) were transfected with plasmids (10 μg) by a calcium phosphate precipitation method with a mammalian transfection kit (Stratagene), following the manufacturer's protocol. Recombinant human TGF-β1 and activin A were iodinated with the chloramine T method (*6, 19*). Cross-linking and immunoprecipitation were done as described (*8*). The samples were analyzed by SDS-PAGE and autoradiography.
26. Rabbit antisera to ALK-5 and TβR-II were made against the intracellular juxtamembrane part of ALK-5 and the COOH-terminal part of TβR-II, respectively (*8*). An antiserum to ActR-II was prepared against the COOH-terminal part of the ActR-II protein (*20*). Antisera against ALKs 1, 2, 3, 4, and 6 were raised against synthetic peptides corresponding to the amino acid sequences of the intracellular juxtamembrane parts of ALKs: ALK-1 peptide, amino acids 145 to 166; ALK-2 peptide, amino acids 151 to 172; ALK-3 peptide, amino acids 181 to 202; ALK-4 peptide, amino acids 153 to 171; and ALK-6 peptide, amino acids 151 to 168. The peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Behring) with glutaraldehyde (*21*), mixed with Freund's adjuvant, and used to immunize rabbits.
27. The R mutant of Mv1Lu cells (clone 4-2) was transfected with plasmids (10 μg) containing cDNAs for ALKs or a control plasmid by the calcium phosphate precipitation method. Transfected cells were incubated with or without TGF-β1 (16 ng/ml) for 2 hours in serum-free MCDB 104 medium without methionine. Cultures were then labeled with [^{35}S]methionine (40 μCi/ml) for 2 hours. Extracellular matrix proteins were prepared as described (*22*) and analyzed by SDS-PAGE and then by fluorography with Amplify (Amersham).
28. For the generation of PAE–Chim A cells, a plasmid (chim A) containing the extracellular domain and COOH-terminal tail of ActR-II (amino acids −19 to 116 and 465 to 494, respectively) (*6*) and the kinase domain of TβR-II (amino acids 160 to 543) (*5*) was constructed and transferred into pcDNA I/neo (Invitrogen). PAE cells were stably transfected with the chim A plasmid by electroporation, and cells expressing the chim A protein were established as described (*8*).

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FGF-2: Apical Ectodermal Ridge Growth Signal for Chick Limb Development

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The apical ectodermal ridge permits growth and elongation of amniote limb buds; removal causes rapid changes in mesodermal gene expression, patterned cell death, and truncation of the limb. Ectopic fibroblast growth factor (FGF)–2 supplied to the chick apical bud mesoderm after ridge removal will sustain normal gene expression and cell viability, and allow relatively normal limb development. A bioassay for FGFs demonstrated that FGF-2 was the only detectable FGF in chick limb bud extracts. By distribution and bioactivity, FGF-2 is the prime candidate for the chick limb bud apical ridge growth signal.

The developing chick limb serves as an experimental system to understand pattern formation (*1*). The limb bud is composed of mesoderm and overlying ectoderm. Capping nearly the entire apex of the bud is a pseudostratified columnar epithelium called the apical ectodermal ridge, which is spatially limited by the simple cuboidal dorsal and ventral limb bud epithelia (*2*). If the ridge is removed surgically it fails to regenerate. Subsequently, only those parts of the limb already determined will develop (*3*). It has been proposed that the ridge, in a permissive way and without direct cell contact (*4*), maintains the mesodermal cells about 200 μm beneath it in a rapidly proliferating and undifferentiated region called the progress zone (*5*). When cells

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