Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells

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The molecular mechanisms controlling inductive events leading to the specification and terminal differentiation of cardiomyocytes are still largely unknown. We have investigated the role of Cripto, an EGF-CFC factor, in the earliest stages of cardiomyogenesis. We find that both the timing of initiation and the duration of Cripto signaling are crucial for priming differentiation of embryonic stem (ES) cells into cardiomyocytes, indicating that Cripto acts early to determine the cardiac fate. Furthermore, we show that failure to activate Cripto signaling in this early window of time results in a direct conversion of ES cells into a neural fate. Moreover, the induction of Cripto activates the Smad2 pathway, and overexpression of activated forms of type I receptor ActRIB compensates for the lack of Cripto signaling in promoting cardiomyogenesis. Finally, we show that Nodal antagonists inhibit Cripto-regulated cardiomyocyte induction and differentiation in ES cells. All together our findings provide evidence for a novel role of the Nodal/Cripto/Alk4 pathway in this process.

Introduction

Specification of vertebrate cardiac mesoderm is the first developmental step leading to heart formation occurring concurrently with gastrulation (Fishman and Chien, 1997). The formation of a vertebrate heart from an amorphous field of precursor cells takes place through multiple developmental steps, such as determination of the cardiac field in the mesoderm, differentiation of cardiac precursor cells into cardiomyocytes, and heart morphogenesis (Olson and Srivastava, 1996). No other early differentiation event is more vital for survival; it must occur in a short time to provide a working circulation system for the rapidly growing embryo (Rosenthal and Xavier-Neto, 2000). Precursor cells, destined for the heart, originate in the lateral epiblast and migrate through the primitive streak to emerge as cardiogenic mesoderm already specified as distinct cardiac lineages. Bilateral fields of cardiogenic mesoderm continue to migrate rostrally and eventually join at their anterior boundaries to form the cardiac crescent, where they commit to cardiac fate (Rosenthal and Xavier-Neto, 2000). The understanding of early

cardiogenesis is of particular interest as cardiomyocyte loss in mammals is largely irreversible and frequently underlies the diminished cardiac function associated with heart diseases (Gepstein, 2002). Recently, some of the secreted factors required for cardiogenesis have been identified (McFadden and Olson, 2002). Members of the bone morphogenetic protein (BMP), Wnt, and EGF-CFC families have been implicated in vertebrate myocardial development. Zebrafish with mutations in the BMP2 homologue gene *swirl* and one-eyed pinhead (the zebrafish member of the vertebrate EGF-CFC family) exhibit severe defects in myocardial differentiation and reduced expression of two early markers of the myocardial precursors Nkx2.5 and GATA5 (Reiter et al., 2001). Results obtained in *Xenopus* and chick indicate that BMP signals from the endoderm induce cardiomyocyte fate, whereas Wnt-mediated signals from the underlying neural tube and notochord suppress cardiomyocyte specification (Schultheiss et al., 1997; Marvin et al., 2001; Tzahor and Lassar, 2001). It has been hypothesized that cardiac muscle cell specification is likely to depend on the location and

duration of signals governing more general developmental decisions in the early embryo (Rosenthal and Xavier-Neto, 2000). In this scenario, the mouse *cripto* gene, the founding member of the EGF-CFC family, appeared to have a crucial role. In mouse embryos, the *cripto* expression profile is associated with the developing heart structures and is detected first in the precardiac mesoderm (Dono et al., 1993). Later on, at 8.5 dpc, *cripto* expression is found in the ventriculus, before being specifically restricted, at 9.5 dpc, to the truncus arteriosus of the developing heart (Dono et al., 1993). Notably, mouse *cripto* mutants exhibit defects in myocardial development, as evidenced by the absence of expression of terminal myocardial differentiation genes such as α-myosin heavy chain (αMHC) and myosin light chain 2v (MLC2v) (Ding et al., 1998; Xu et al., 1999). Accordingly, by using embryoid bodies (EBs) derived from Cripto$^{-/-}$ ES cells, it has been shown that *cripto* is essential for cardiomyocyte induction and differentiation (Xu et al., 1998). However, how *cripto* functions to regulate cardiogenesis is still unknown. To study this process, we took advantage of embryonic stem (ES) cells, which have been widely used as a model system of cardiogenesis, proven to be a powerful tool to study early events of cardiac induction (Doetschman et al., 1993; Monzen et al., 2001, 2002; Boheler et al., 2002). To create a system in which we could manipulate Cripto activity, we developed an assay in which recombinant Cripto protein restored cardiomyocyte differentiation in Cripto$^{-/-}$ ES cells. This approach allowed us to define the dynamics of Cripto signaling required for differentiation of cardiac precursor cells. We showed that Cripto is required in a precise moment during differentiation, after which it fails to specify the cardiac lineage. Moreover, we found that the absence of Cripto signaling in this early acting window of time resulted in a direct conversion of Cripto$^{-/-}$ EB–derived cells into a neural fate. This observation suggests that Cripto inhibits mammalian neuralization and supports the hypothesis that a default model for neural specification is operating in ES cells. Furthermore, we show that Cripto protein activates the Smad2 pathway during cardiomyocyte induction and, moreover, that overexpression of an activated form of type I receptor ActRIB restored the ability of Cripto$^{-/-}$ ES cells to differentiate into cardiomyocytes. Taken together, our results indicate that Cripto participates in heart development, regulating early events that lead to cardiac specification, and highlight a novel role for the Nodal/Cripto/Alk4 pathway in cardiomyogenesis.

Results

Secreted Cripto retains its ability to rescue cardiomyocyte differentiation

Previous data on cultured ES cells lacking *cripto* have revealed an essential role of *cripto* for contractile cardiomyocyte formation. Cripto$^{-/-}$ ES cells selectively lose the ability to form beating cardiomyocytes, a process that can be rescued by expression of Cripto (Xu et al., 1998). As Cripto is a GPI-anchored membrane protein, we first determined if a secreted form of Cripto could restore cardiomyocyte differentiation in Cripto$^{-/-}$ ES cells (Fig. 1). To this end, we overexpressed a secreted derivative of Cripto, which lacks the
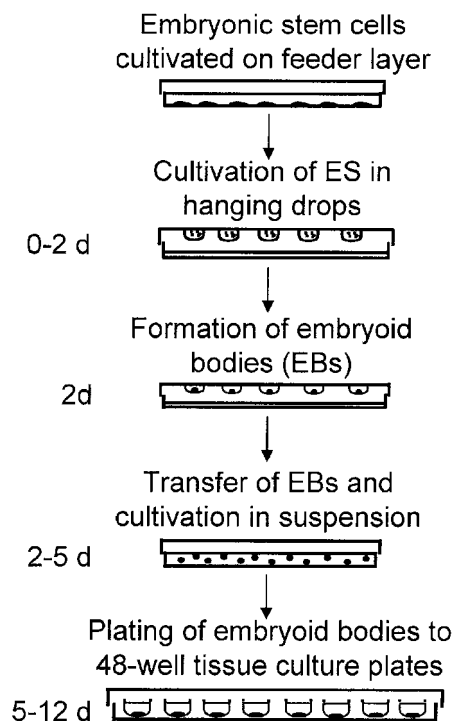

Figure 1. **Schematic representation of the experimental protocol used for ES cell differentiation into cardiomyocytes (adapted from Maltsev et al., 1993).**

hydrophobic COOH terminus region required for membrane anchorage (Minchiotti et al., 2000), in Cripto$^{-/-}$ ES cells and compared its activity to that of wild-type (wt) Cripto (Fig. 2 A). A pooled population of cells selected for resistance to puromycin were examined for the number of EBs containing beating areas, from day 8 to 12 of the in vitro differentiation assay. Spontaneous rhythmic contractile myocytes were observed in Cripto$^{-/-}$ ES cells expressing either the membrane-anchored or the secreted Cripto protein (Fig. 2 B). Moreover, similar results were obtained by expressing a secreted Cripto protein that lacks the NH$_2$ terminus region (EGF-CFC; Fig. 2, A and B), thus indicating that not only membrane anchorage is dispensable for activity, but that the EGF-CFC domain alone is sufficient for Cripto activity in the cardiogenic induction. Previous data have shown that a refolded synthetic Cripto peptide containing the EGF-like domain had mitogenic and branching activity for mammary cell lines (Salomon et al., 1999). We thus went on to define whether the Cripto EGF-like domain alone was able to induce cardiogenesis similar to the EGF-CFC peptide. Two *cripto* cDNA deletion derivatives encoding either the EGF-like domain retaining the NH$_2$ terminus region (EGF long) or just the EGF domain (EGF short; Fig. 2 A) were generated. No beating areas were observed in EBs derived from Cripto$^{-/-}$ ES cells expressing either EGF long or EGF short peptide (Fig. 2 B), thus indicating that both EGF and CFC domains of Cripto are essential for cardiogenic induction. Western blot analysis showed that the EGF long and EGF short peptides were produced and secreted as efficiently as EGF-CFC (Fig. 2 C and not depicted), thus demonstrating that their inability to rescue the mutant phe-
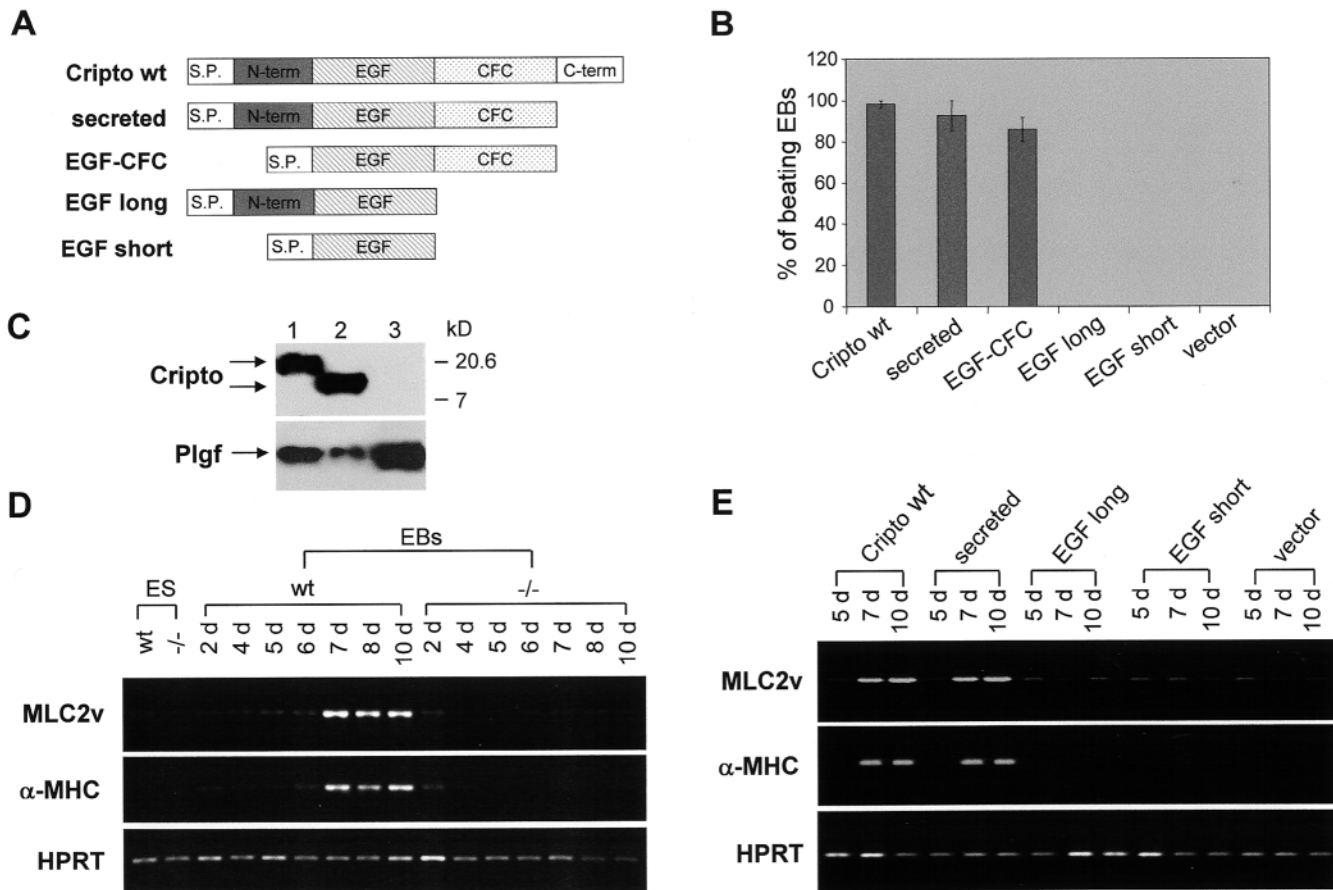
Figure 2. **Functional dissection of Cripto.** (A) Schematic representation of *cripto* cDNA derivatives. S.P., signal peptide. (B) Determination of minimal domains required for Cripto activity in cardiomyocyte differentiation. Either wt or deleted *cripto* mutant derivatives were transfected into Cripto$^{-/-}$ ES cells; empty vector was used as control. The percentage of EBs with rhythmically contracting areas detectable by light microscopy was scored on day 8 to 12. Data are representative of at least two independent experiments. (C) Western blot analysis of conditioned media from 293EBNA cells transfected with *cripto* cDNA deletion derivatives. Cells were cotransfected with *Plgf* expression vector as an internal control (see Materials and methods). Lane 1, EGF-CFC; lane 2, EGF long; lane 3, vector. The molecular mass of protein standards is indicated (kD). (D) Expression of cardiac-specific genes MLC2v and αMHC during in vitro differentiation of either wt or Cripto$^{-/-}$ ES cells. RT-PCR was performed on RNA extracted from either undifferentiated ES cells or EBs throughout a differentiation period of 10 d (days 2–10). HPRT gene expression was analyzed as an internal control. (E) RNA expression levels of MLC2v and cardiac αMHC genes during in vitro differentiation of Cripto$^{-/-}$ ES cells overexpressing either wt or *cripto* deletion mutants. RNA was harvested at days 5, 7, and 10 of the differentiation protocol and subjected to RT-PCR. Empty vector was used as a negative control. HPRT gene expression was analyzed as an internal control. The results are representative of two independent differentiation programs.

notype was not due to a difference in protein expression level. To support the morphological data observed, we examined the expression levels of the cardiac-specific αMHC and MLC2v, two major contractile proteins of cardiomyocytes. As expected, expression of both the αMHC and MLC2v genes was induced in wt ES cells but not in Cripto$^{-/-}$ cells from day 7 of in vitro differentiation (Fig. 2 D). Importantly, the expression pattern of αMHC and MLC2v genes in wt ES cells was reproduced in Cripto$^{-/-}$ cells expressing either wt Cripto or the secreted derivative, but not in cells expressing either EGF long or EGF short peptides (Fig. 2 E).

Timing and duration of Cripto activity in cardiomyocyte differentiation

To gain further insight into the functional role of Cripto in cardiogenic induction and differentiation, we first examined the timing of Cripto expression during ES cell differentia-

tion. Western blot analysis performed with anti-Cripto antibodies on lysates from both wt and Cripto$^{-/-}$ ES cells revealed that Cripto was detectable as early as day 0 and peaked in expression by day 4 in wt EBs (Fig. 3). Importantly, the transient nature of Cripto accumulation suggested that its activity might be required at a defined step in cardiomyocyte differentiation. The time window of Cripto action could not be adequately investigated by means of transfection assays. Therefore, to directly address this issue, a recombinant soluble Cripto protein was used in which the hydrophobic COOH terminus was replaced by a 6xHis epitope (Cripto-His; Minchiotti et al., 2001). Based on our observation that secreted Cripto protein is able to promote cardiogenesis when expressed in the Cripto$^{-/-}$ ES cells (Fig. 2 B), experiments were performed where Cripto signaling was reconstituted by addition of recombinant secreted Cripto protein directly to the cells (Fig. 4). Addition of Cripto during the 0–2-d interval effectively restored the dif-
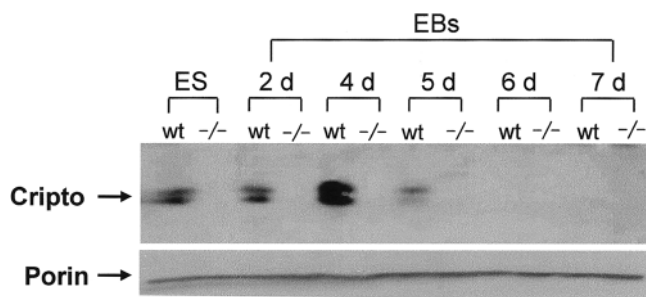
Figure 3. **Cripto expression profile during the in vitro differentiation of ES cells.** Total lysates of either undifferentiated ES cells or EBs at different days of differentiation (2–7 d), derived from either RI (wt) or DE7 (Cripto$^{-/-}$) ES cells, were collected in lysis buffer and analyzed by Western blot using a polyclonal anti-Cripto antiserum (Minchiotti et al., 2000). Data were normalized to the expression level of Porin.

ferentiation ability of Cripto$^{-/-}$ ES cells. Addition at later time points resulted in dramatically reduced cardiomyocyte differentiation (Fig. 4 A). Comparable results were obtained with two independent Cripto$^{-/-}$ ES clones (DE7 and DE14; Xu et al., 1998), thus excluding any phenotype difference due to clonal variation (Fig. 4 A). All together, these data indicated that stimulation in trans with soluble Cripto protein was fully efficient in promoting cardiomyocyte induction and differentiation and, more interestingly, defined exactly when Cripto activity was required to promote specification of the cardiac lineage. Furthermore, to define the optimal concentration of Cripto required to promote cardiogenesis, increasing amounts of purified recombinant Cripto protein were added directly to the culture medium of 2-d-old Cripto$^{-/-}$ EBs from either DE7 or DE14 cell lines for 24 h (Fig. 4 B). Increasing amounts of recombinant

Cripto resulted in enhanced differentiation efficiency (Fig. 4 B), thus indicating that Cripto-mediated cardiogenic induction was dose dependent.

Having shown that the timing and dose of Cripto signaling activation were crucial to promote cardiomyocyte induction and differentiation, we thus went on to define whether the duration of Cripto signaling was crucial for its biological response. 2-d-old EBs from DE7 or DE14 Cripto$^{-/-}$ ES cells were treated with 10 μg/ml of recombinant Cripto for various lengths of time, washed to remove unbound Cripto, and then cultured for the remaining days. An effective Cripto response required a minimum induction of 24 h, while shorter inductions showed markedly reduced activity (Fig. 4 C). Taken together, our data demonstrated that the amount, timing, and duration of Cripto signaling were all crucial factors to achieve cardiogenic induction and differentiation.

Direct conversion of Cripto$^{-/-}$ EB–derived cells into a neural fate

Our observation that initiation of Cripto signaling in an early acting window of time is crucial for priming differentiation of ES cells to cardiac fate prompted us to gain further insight into the functional role of Cripto at an early phase of ES cell differentiation. Interestingly, when Cripto$^{-/-}$ EBs were plated onto an adhesive substrate, a population of cells with a neuron-like morphology was observed that produced a network surrounding the aggregates. This characteristic morphology was never observed either in wt EBs or in Cripto$^{-/-}$ EBs treated with effective doses of Cripto protein. To confirm that those cells were indeed neurons, immunofluorescence analysis was performed on both wt and Cripto$^{-/-}$ EBs, by using antibodies that recognize the neuron-specific form of class βIII tubu-
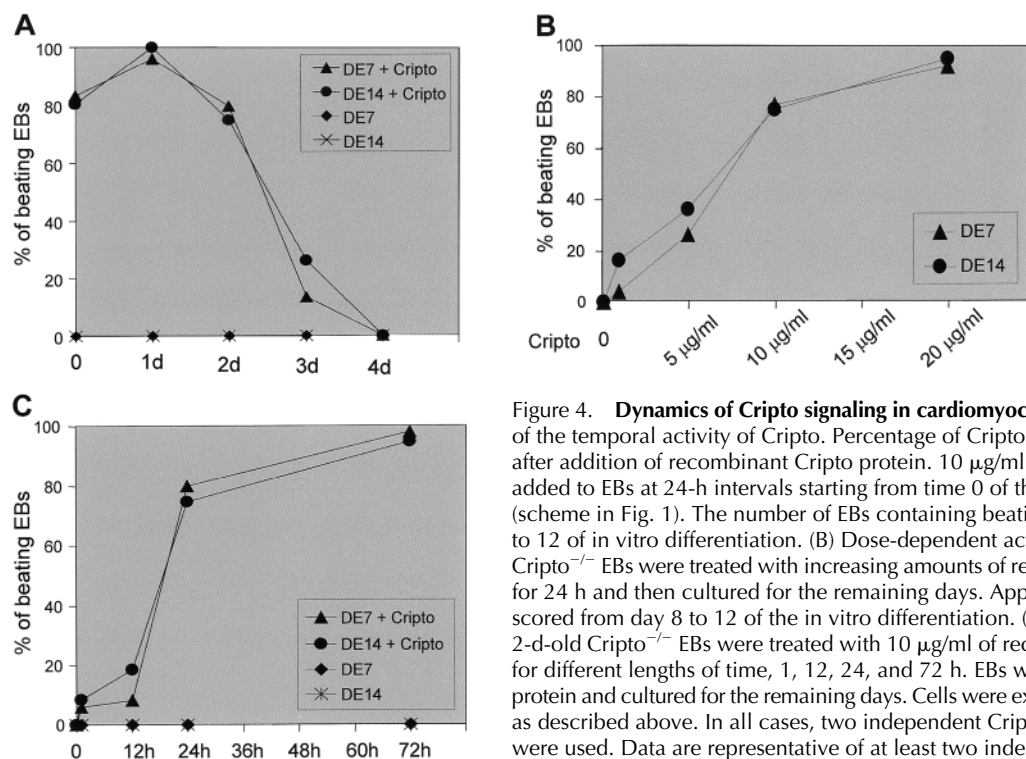

Figure 4. **Dynamics of Cripto signaling in cardiomyocyte differentiation.** (A) Definition of the temporal activity of Cripto. Percentage of Cripto$^{-/-}$ EBs containing beating areas after addition of recombinant Cripto protein. 10 μg/ml of soluble Cripto protein was added to EBs at 24-h intervals starting from time 0 of the in vitro differentiation assay (scheme in Fig. 1). The number of EBs containing beating areas was scored from day 8 to 12 of in vitro differentiation. (B) Dose-dependent activity of Cripto protein. 2-d-old Cripto$^{-/-}$ EBs were treated with increasing amounts of recombinant soluble Cripto protein for 24 h and then cultured for the remaining days. Appearance of beating areas was scored from day 8 to 12 of the in vitro differentiation. (C) Duration of Cripto signaling. 2-d-old Cripto$^{-/-}$ EBs were treated with 10 μg/ml of recombinant soluble Cripto protein for different lengths of time, 1, 12, 24, and 72 h. EBs were then washed to remove the protein and cultured for the remaining days. Cells were examined for cardiac differentiation as described above. In all cases, two independent Cripto$^{-/-}$ ES clones (DE7 and DE14) were used. Data are representative of at least two independent experiments.
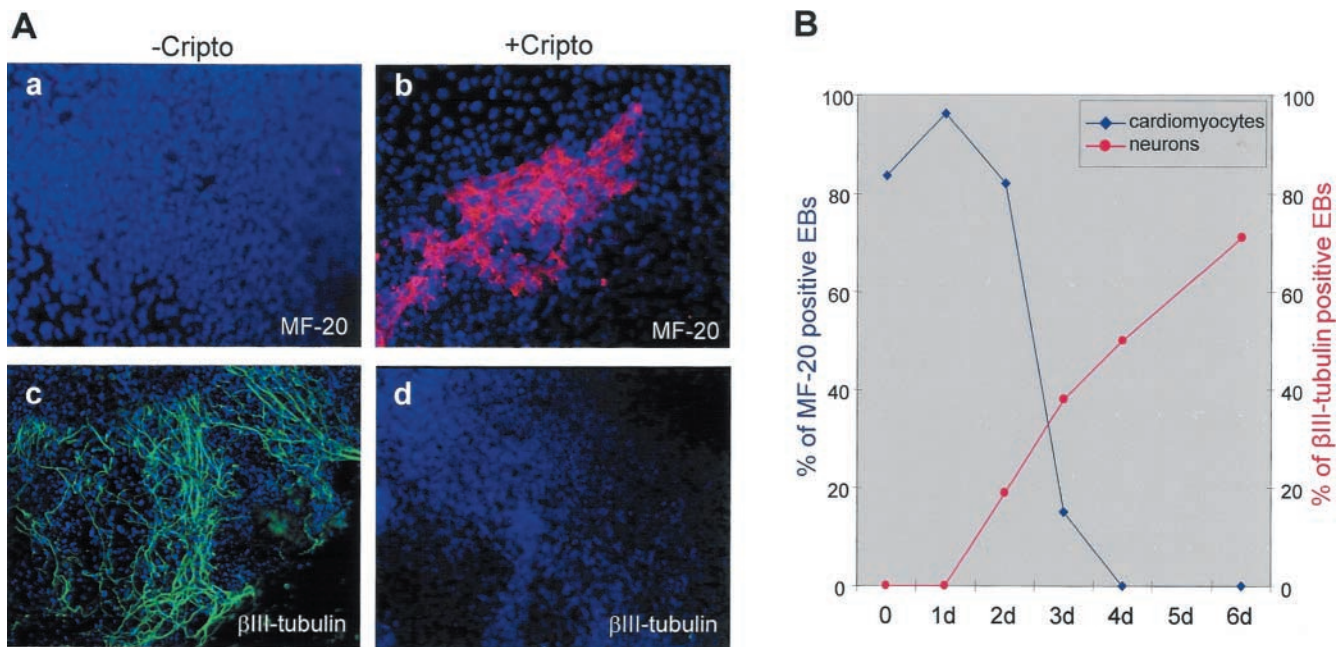
Figure 5. **Cripto promotes cardiomyocyte differentiation and inhibits neural differentiation of ES cells according to the timing of exposure.**
(A) Cardiomyocyte versus neuronal differentiation of Cripto$^{-/-}$ EBs as revealed by indirect immunofluorescence. 2-d-old Cripto$^{-/-}$ EBs, derived from DE7 cell line, were either left untreated (a and c) or treated for 24 h with 10 μg/ml of recombinant Cripto protein (b and d). On day 12 of in vitro differentiation, expression of either sarcomeric myosin or βIII-tubulin was revealed by immunofluorescence using anti–MF-20 (red, a and b) or βIII-tubulin (green, c and d) antibodies, respectively. Data are representative of at least two independent experiments. Comparable results were obtained with Cripto$^{-/-}$ DE14 ES cell line. (B) Cardiomyocyte versus neuronal differentiation of Cripto$^{-/-}$ EB–derived cells depends on the timing of exposure to Cripto. Percentage of Cripto$^{-/-}$ EBs stained for βIII-tubulin (red plot) or MF-20 (blue plot) after addition of recombinant Cripto protein at different time points. 10 μg/ml of recombinant Cripto protein was added to EBs at 24-h intervals starting from time 0 of the in vitro differentiation assay. On day 12 of in vitro differentiation, EBs were stained for either βIII-tubulin or MF-20 antibodies. Data are representative of two independent experiments.

lin. These antibodies stained clusters of cells in Cripto$^{-/-}$ EBs, revealing the presence of a dense network of neurons (Fig. 5 A). Neurons were detected in 71% of Cripto$^{-/-}$ EBs, whereas βIII-tubulin–positive cells were never detected in both wt EBs and rescued Cripto$^{-/-}$ EBs that, on the contrary, showed extensive areas of MF-20–positive cardiomyocytes (Fig. 5 A). To gain insight into this issue, we used our controlled differentiation assay to modulate Cripto signaling and to eventually score EB-derived cells for either cardiomyocyte or neuron differentiation, by using morphological criteria as well as immunofluorescence analysis. Addition of Cripto protein during the 0–2-d interval rescued, as expected, the cardiac phenotype of Cripto$^{-/-}$ ES cells (Fig. 5 B), but also resulted in a dramatic inhibition of neural differentiation (Fig. 5 B). Conversely, addition of recombinant Cripto at later time points (i.e., 3–6-d interval) resulted in progressive impairment of cardiac differentiation (see previous paragraph and Fig. 5 B) and, at the same time, increased competence of the EB-derived cells to acquire a neural phenotype, resulting in close to 70% of Cripto$^{-/-}$ EBs that show extensive areas of βIII-tubulin–positive cells. All together our results support the hypothesis that Cripto signaling represses neural differentiation in ES cells and, moreover, show that the restricted time window of Cripto signaling required to achieve proper terminal cardiac differentiation of Cripto$^{-/-}$ ES cells correlates with the competence window for those cells to become committed to a neuronal phenotype.

Cripto activates a Smad2 pathway associated with cardiomyocyte differentiation

Findings in mice, *Xenopus*, and zebrafish point to a strong functional link between the EGF-CFC proteins and TGFβ ligand Nodal (Shen and Schier, 2000; Adamson et al., 2002). Accordingly, recent studies have shown that Cripto can associate with type I receptor ActRIB (Alk4) and can form a complex together with Nodal and type II receptor ActRIIB (Reissmann et al., 2001; Yeo and Whitman, 2001; Bianco et al., 2002; Yan et al., 2002). Activation of Smad proteins by phosphorylation is a universal signal transduction event following activation of Alk receptors. To ask whether Cripto activates the Smad2 pathway during cardiomyocyte induction and differentiation, 2-d-old Cripto$^{-/-}$ EBs were starved in low serum for 3 h and then stimulated with recombinant soluble Cripto protein for 30, 60, or 120 min. Western blot analysis revealed that phosphorylation of Smad2 significantly increased after treatment with recombinant Cripto (Fig. 6). Smad2 phosphorylation was detectable already after 30-min treatment, persisting at comparable levels even after prolonged exposure to Cripto protein. An anti-Smad2/3 antibody applied to the same blot was used to normalize for total amount of protein (Fig. 6). In vitro studies on mammary cell lines have suggested that Cripto is involved in the Ras/Raf/MEK/MAPK pathway (Salomon et al., 1999). When we looked for activation of the MAP kinase ERK by using an anti–phospho-ERK antibody, recombinant Cripto was unable to activate MAP kinase (unpub-
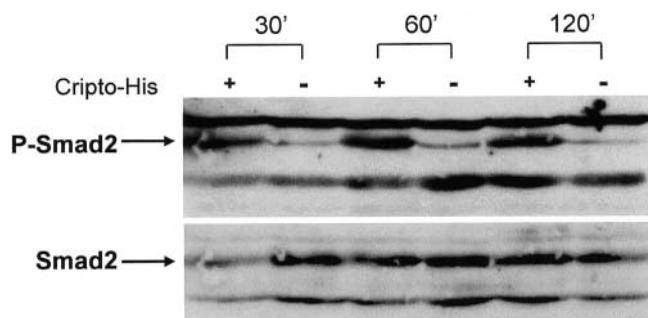
Figure 6. **Activation of Smad2 in Cripto$^{-/-}$ cell aggregates treated with recombinant Cripto protein.** 2-d-old Cripto$^{-/-}$ EBs were serum starved for 3 h and then treated with 10 μg/ml of recombinant Cripto protein for 30', 60', or 120' or left untreated, as indicated. Smad2 activation was detected by Western blot analysis using anti–phospho-Smad2 antibody. Levels of total Smad2 were also compared.

lished data); thus indicating that the Smad2 pathway was selectively activated during cardiomyocyte induction and differentiation induced by Cripto.

To our knowledge, no data are available on the expression profile of all components of the Alk4/ActRIIB/Nodal complex during the differentiation of ES cells; thus, we first measured by RT-PCR the expression of Nodal, Alk4, and ActRIIB in EBs derived from both wt and Cripto$^{-/-}$ ES cells. Nodal, Alk4, and ActRIIB were expressed in all analyzed stages (Fig. 7 A). If Cripto signaling in cardiomyocyte differentiation acts via the Alk4 receptor, overexpression of a constitutively active type I receptor would be expected to compensate for the lack of Cripto signaling in promoting cardiomyocyte differentiation. We overexpressed in Cripto$^{-/-}$ ES cells the wt or constitutively activated form (ca) of either human HA-tagged Alk4 or its zebrafish counterpart Taram-A (Renucci et al., 1996). Type I receptor serine/threonine kinases can be activated in a ligand- and type II receptor–independent manner by replacing an acidic residue for a specific threonine within the juxtamembrane region of the intracellular domain, a segment known to be involved in kinase regulation (Wieser et al., 1995). Overexpression of either Alk4 ca or

Table I. **Percentage of beating EBs from Cripto$^{-/-}$ ES cells transfected with either wt or ca form of human Alk4 or zebrafish Taram-A receptors**

Cells	Construct	EBs scored	% of beating EBs
DE7	None	70	0
DE7	Cripto wt	50	96.6
DE7	Alk4 wt	76	0
DE7	Alk4 ca	50	16.0
DE7	Taram-A wt	55	0
DE7	Taram-A ca	64	45.0
DE7	Empty vector	56	0
DE14	None	80	0
DE14	Cripto wt	54	94.4
DE14	Taram-A wt	50	1.9
DE14	Taram-A ca	51	62.2
DE14	Empty vector	60	0

Data are representative of at least two independent experiments. DE7 and DE14 are two independent Cripto$^{-/-}$ clones.
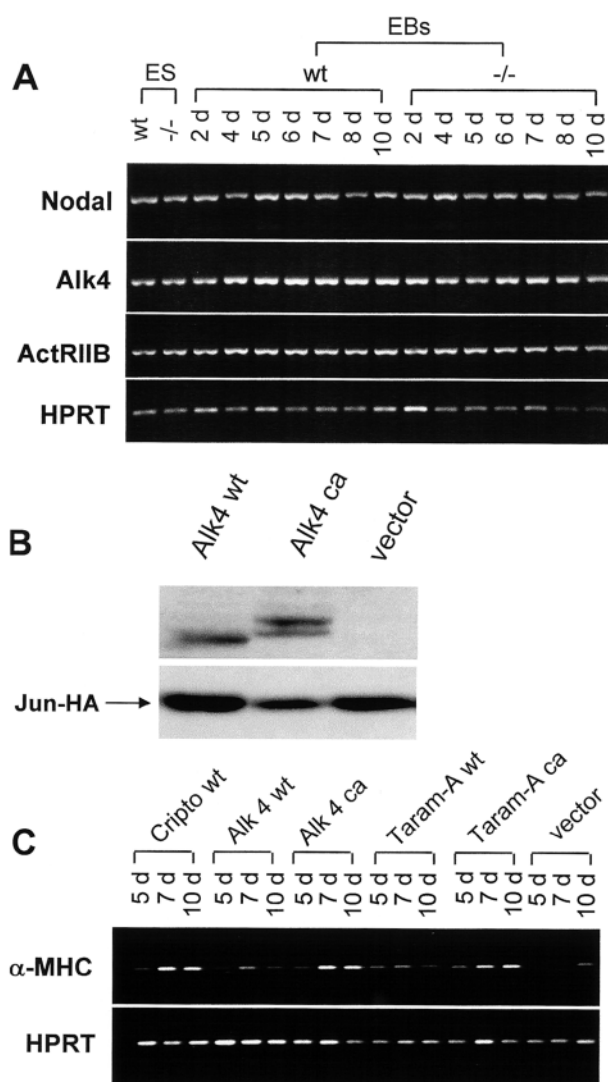

Figure 7. **Expression profile of Nodal, Alk4, and ActRIIB during cardiomyocyte differentiation and their effects on cardiac induction.** (A) RNA expression levels of Nodal, Alk4, and ActRIIB genes during in vitro differentiation of ES cells. RT-PCR analysis was performed on RNA extracted from either undifferentiated ES or EBs (either wt or Cripto$^{-/-}$) throughout a differentiation period of 10 d (days 2–10). HPRT gene was used as an internal control. (B) Western blot analysis of total lysates from 293EBNA cells transfected with either wt or ca form of HA-tagged human Alk4. Cells were cotransfected with Jun-HA expression vector as an internal control. A monoclonal anti-HA antibody was used to detect protein levels. (C) RNA expression profile of the αMHC gene during differentiation of Cripto$^{-/-}$ ES cells (days 5, 7, and 10) overexpressing wt and activated forms of either Alk4 or Taram-A. HPRT gene was used as an internal control.

Taram-A ca partially restored the ability of Cripto$^{-/-}$ ES cells to differentiate into cardiomyocytes (Table I). On the contrary, overexpression of the wt receptors, either Taram-A or Alk4, had no significant activity despite their similar expression levels (Fig. 7 B). Furthermore, addition of recombinant Cripto to Alk4 ca–expressing cells restores cardiomyocyte differentiation close to controls (Table II). In accordance with the morphological data, expression of the αMHC gene was only detected in Cripto$^{-/-}$ ES cells expressing the activated form of the receptors (Fig. 7 C). Altogether, our results pro-

Table II. **Percentage of beating EBs from transfected Cripto$^{-/-}$ ES cells**

Construct	Protein	EBs scored	% of beating EBs
Alk4 ca	None	50	16.0
Alk4 ca	Cripto[a]	87	87.3
Empty vector	None	49	0
Empty vector	Cripto[a]	60	96.6

[a]2-d-old EBs were treated with 10 μg/ml of recombinant Cripto for 3 d.

vide an intriguing link between Alk4/Smad pathway activation by Cripto and cardiomyocyte development.

Analysis of Cripto mutants identifies key residues both in the EGF and in the CFC domain

We showed above that the EGF-CFC domain is sufficient to promote cardiogenic induction when overexpressed in Cripto$^{-/-}$ ES cells, whereas the EGF domain alone is unable to rescue the biological activity. To determine the contribution of the EGF and CFC domains, single amino acid substitutions were introduced into the *cripto* cDNA (Fig. 8 A), and the activity of the corresponding mutant proteins was compared with the wt in the cardiomyocyte assay. Although each mutant was expressed at levels comparable to wt Cripto (Fig. 8 B), three of them were completely inactive or showed a strongly reduced activity in our assay (Table III). Similar results were obtained with two independent Cripto$^{-/-}$ ES clones (Table III). To support the observed morphological data, the expression of the αMHC and the MLC2v genes was examined by RT-PCR on total RNA prepared from EBs derived from Cripto$^{-/-}$ ES cells overexpressing Cripto mutant derivatives (Fig. 8 C). Expression of

Table III. **Percentage of beating EBs from Cripto$^{-/-}$ ES cells transfected with either wt or Cripto mutant derivatives**

Cells	Construct	EBs scored	% of beating EBs
DE7	None	97	0
DE7	Cripto wt	56	98.2
DE7	N63I	54	91.5
DE7	G71N	54	0
DE7	T72A	62	90.3
DE7	S77A	60	95.0
DE7	F78A	47	42.5
DE7	F78W	60	95.0
DE7	H104A	56	89.3
DE7	W107G	57	7.6
DE7	R116G	49	80.0
DE7	L122N	103	92.0
DE7	Empty vector	65	0
DE14	None	85	0
DE14	Cripto wt	54	94.4
DE14	G71N	49	0
DE14	F78A	45	66.0
DE14	W107G	57	30.5
DE14	Empty vector	71	0

Data are representative of at least two independent experiments. DE7 and DE14 are two independent Cripto$^{-/-}$ clones.

αMHC and MLC2v genes was either absent or reduced in cells overexpressing G71N, F78A, or W107G *cripto* mutants, whereas it was restored in Cripto$^{-/-}$ cells transfected with wt *cripto*. Together these data show that critical amino acid residues are located in both EGF and CFC domains, thus indicating the requirement of both domains for Cripto activity in cardiogenic induction.
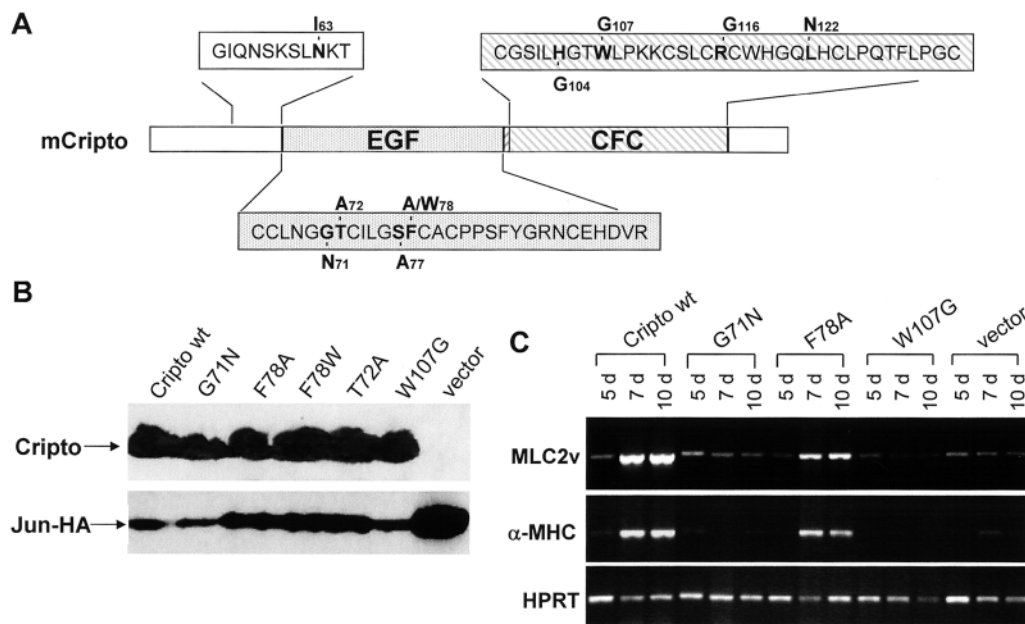

Figure 8. **Identification of Cripto key residues required for cardiac induction and differentiation.** (A) Schematic representation of wt and mutant Cripto derivatives. (B) Western blot analysis of total lysates from 293EBNA cells transfected with either wt or mutant *cripto* derivatives. Jun-HA expression vector was cotransfected as an internal control. Either polyclonal anti-Cripto or monoclonal anti-HA antibodies were used to detect protein levels. (C) RNA expression levels of the cardiac αMHC and MLC2v genes during in vitro differentiation of Cripto$^{-/-}$ ES cells (days 5, 7, and 10) overexpressing either wt or mutant *cripto* derivatives. Expression level of HPRT gene was analyzed as an internal control.
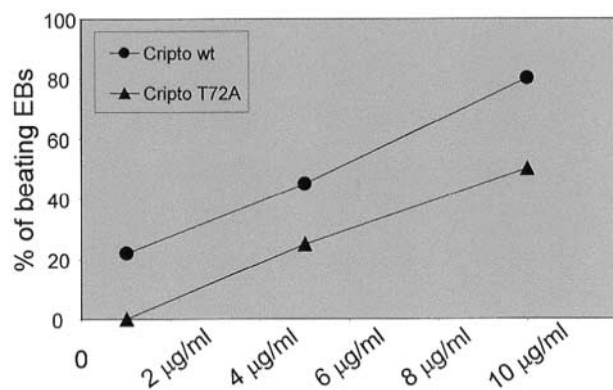
Figure 9. **Modulation of Cripto activity by O-fucosylation.** Dose-dependent activity of T72A mutant Cripto compared with wt Cripto as assayed in cardiomyocyte differentiation assay. 2-d-old Cripto$^{-/-}$ EBs were treated with increasing amounts of either recombinant soluble T72A mutant or wt Cripto protein for 24 h and then cultured for the remaining days. Appearance of beating areas was scored from day 8 to 12 of the in vitro differentiation. Data are representative of two independent experiments.

Recent reports have shown that Cripto is modified by the addition of sugar residues. N-linked glycosylation was shown to affect Cripto biological activity in the zebrafish assay (Minchiotti et al., 2001). More recently, an O-linked fucosylation of Cripto has been reported to be required for Cripto signaling activity in cotransfection assay in mammalian cells (Schiffer et al., 2001; Yan et al., 2002). To assess if posttranslational modifications were required for Cripto activity in cardiogenic induction, two alanine substitutions were generated, corresponding to either the N-glycosylation site (N63I) or the O-linked fucosylation site (T72A). The activities of the corresponding mutant proteins were tested in the differentiation assay and compared with wt Cripto. Based on the percentage of EBs containing beating areas, both mutant proteins had comparable ability in promoting cardiomyocyte differentiation, compared with wt Cripto (Table III), thus suggesting that addition of sugar residues was not strictly required for Cripto activity in ES cells.

However, a role of these modifications in the modulation of Cripto signaling might be masked in our assay due to overexpression of the proteins. To overcome this limitation, we purified a recombinant Cripto T72A mutant protein from conditioned medium of transfected 293 cells, and its activity was compared with the wt Cripto. When used in the cardiomyocyte differentiation assay, the Cripto T72A mutant protein resulted in close to a 30% reduction in the numbers of Cripto$^{-/-}$ EBs displaying beating cardiomyocytes, compared with the wt Cripto (Fig. 9). A similar reduction was observed when using Cripto T72A in the Smad2 phosphorylation assay, indicating that doses higher than those used for wt Cripto were required to achieve equivalent induction (unpublished data).

Nodal antagonists inhibit Cripto activity in cardiomyogenesis

To gain direct evidence that Nodal signaling is indeed required to support Cripto-regulated cardiac induction and differentiation in ES cells, we sought to determine whether
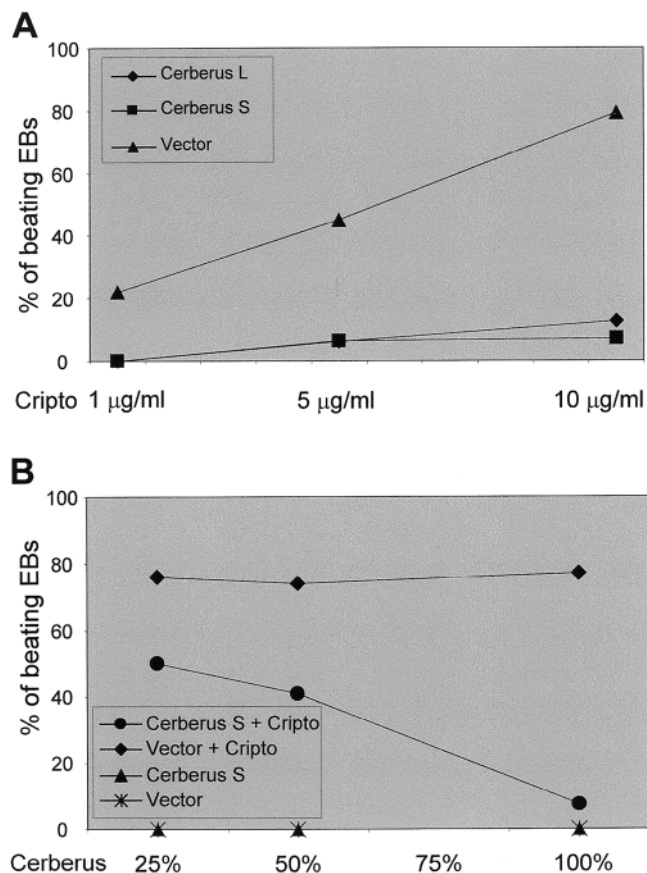

Figure 10. **Exposure to Cerberus inhibits Cripto activity in cardiomyocyte differentiation assay.** (A) Cerberus inhibits Cripto-dependent cardiomyocyte differentiation of Cripto$^{-/-}$ EBs. 2-d-old Cripto$^{-/-}$ EBs were cultured for 24 h in the presence of 100% (vol/vol) of media supernatant from 293T cells transiently expressing either Cerberus, Cerberus-S, or empty vector as control, in the presence of increasing amounts of recombinant Cripto protein. The number of Cripto$^{-/-}$ EBs containing beating areas was scored from day 8 to 12 of in vitro differentiation. Data are representative of two independent experiments. (B) Inhibition of Cripto by Cerberus-S is dose dependent. 2-d-old Cripto$^{-/-}$ EBs were cultured for 24 h with 10 µg/ml of soluble Cripto protein in the presence of increasing amounts (vol/vol) of media from 293T cells transiently expressing Cerberus-S. Cells were examined for cardiac differentiation as described above. Data are representative of two independent experiments.

inhibition of Nodal signaling might interfere with Cripto ability to promote cardiomyogenesis. To directly address this point, 2-d-old Cripto$^{-/-}$ EBs were treated with increasing amounts of recombinant Cripto (1–10 µg/ml) in a media containing the supernatant collected from a transiently transfected 293T cell line producing Cerberus protein. This multifunctional antagonist inhibits Nodal as well as BMP and Wnt signaling. However, a truncated form of Cerberus, named Cerberus-Short (Cerberus-S), is a specific Nodal antagonist (Piccolo et al., 1999). The presence of either Cerberus or Cerberus-S supernatant resulted in a significant inhibition of Cripto ability to prime cardiomyocyte differentiation compared with control supernatant (Fig. 10 A). Furthermore, we treated Cripto$^{-/-}$ EBs with 10 µg/ml of recombinant Cripto in the presence of increasing amounts of Cerberus-S–containing medium,

Table IV. **Percentage of beating EBs from Cripto$^{-/-}$ ES cells transfected with Nodal antagonists**

Construct	Protein	EBs scored	% of beating EBs
Empty vector	None	40	0
Empty vector	Cripto[a]	58	85
Cerberus	None	34	0
Cerberus	Cripto[a]	49	10.3
Cerberus-S	None	36	0
Cerberus-S	Cripto[a]	40	8.3

[a]2-d-old EBs were treated with 10 µg/ml of recombinant Cripto for 3 d.

thus being able to show that inhibition of Cripto activity by Cerberus-S was indeed dose dependent (Fig. 10 B). Finally, as an additional control, we used Cripto$^{-/-}$ ES cells transfected with either Cerberus or Cerberus-S expression vectors, before treatment of the derived EBs with recombinant Cripto. In accord with the results obtained with conditioned media, expression of either Cerberus or Cerberus-S resulted in a significant inhibition of Cripto activity (Table IV). Together, these results show that Cerberus and Cerberus-S can act as effective antagonists of Cripto signaling in ES cell differentiation and provide evidence for a functional role of the Nodal pathway in Cripto-mediated specification of the cardiac lineage.

Discussion

Role of secreted Cripto as a priming factor for cardiomyogenesis

Cripto is a GPI-anchored protein; however, recent data in zebrafish have shown that Cripto protein could be provided in a soluble form to enable proper Nodal signal propagation (Minchiotti et al., 2001). Moreover, previous data on chimeric mouse embryos established from a combination of wt and Cripto$^{-/-}$ ES cells suggested that Cripto acts nonautonomously during development (Xu et al., 1998). Due to the absence of analysis of cellular genetic markers, and because only late-stage chimeric embryos were analyzed, the cell autonomous or nonautonomous activity of Cripto is an issue that still remains unsolved (Rosa, 2002). Expression of different deletion mutant derivatives of *cripto* cDNAs in Cripto$^{-/-}$ ES cells led us to demonstrate that secreted Cripto could efficiently induce cardiogenesis and, moreover, that expression of the EGF-CFC domain was sufficient to restore the ability of Cripto$^{-/-}$ ES cells to differentiate into cardiomyocytes. Furthermore, we showed that the Cripto EGF domain is required, but not sufficient, for cardiomyogenesis. In contrast, it was previously demonstrated that a refolded synthetic human Cripto peptide comprising only the EGF-like domain displayed high affinity binding and mitogenic activity on mammary cell lines (Salomon et al., 1999). Considering the high percentage of amino acid identity in the EGF domain between human and mouse Cripto (Dono et al., 1993), this observation strengthened the hypothesis that there may be divergent Cripto signaling pathways, depending both on different Cripto domains and on specific cell types, that remain to be explored in the future.

Dose and temporal regulation of Cripto signaling in the commitment of ES cells to cardiac fate

EB differentiation is currently considered a powerful model system, reproducing many aspects of in vivo tissue formation during the crucial, but less accessible, stages of mammalian embryogenesis, thus providing access to early cell populations that develop in a normal fashion (Keller, 1995). The timing of initiation of Cripto signaling and the strength and duration of the signal are interdependent variables that have not yet been resolved experimentally, mainly due to the complexity of in vivo analysis. Western blot analysis performed on total lysates, prepared at different times of ES cell in vitro differentiation, showed a regulated accumulation of Cripto protein whose expression was restricted to the very beginning of the differentiation program.

We then used soluble Cripto protein on Cripto$^{-/-}$ ES cells to modulate Cripto signaling and measured its effect on cardiomyocyte differentiation. Kinetic experiments performed by adding recombinant Cripto protein directly to the culture medium of Cripto$^{-/-}$ ES cells demonstrated that stimulation in trans with a soluble Cripto peptide was capable of promoting cardiac induction and, strikingly, revealed an early acting window of time in which the initiation of Cripto signaling is crucial for priming differentiation of ES cells to cardiac fate. Having defined the timing of Cripto signaling, we next examined two more variables, dose and duration of signaling. Either different amounts of Cripto protein for a defined length of time or a high dose of Cripto peptide for various lengths of time was used on Cripto$^{-/-}$ EBs. Both protein dose and signaling duration were crucial parameters. Worth noticing, our results show that transient presence of Cripto is inadequate and that sustained Cripto signaling is strictly required to promote cardiogenesis. We thus propose that Cripto activity at a given time, strength of the signal, and length of the time of exposure are critical parameters for the correct specification and differentiation of the cardiac lineage.

Neural cell fate is established from Cripto$^{-/-}$ EB–derived cells in the absence of retinoic acid

Several studies demonstrated that neural differentiation from ES cells relies upon an aggregation step followed by exposure to specific inducing factors, such as retinoic acid (Bain et al., 1995, 1996; Okabe et al., 1996). Our present findings indicate that in the absence of retinoic acid, Cripto$^{-/-}$ EB–derived cells spontaneously differentiate into neurons. Furthermore, we show that the timing of Cripto signaling required for priming differentiation of cardiac cells resembles the competence window of EB-derived cells to acquire a neural character. All together, our results indicate that Cripto signaling is strictly required in an early acting window to negatively regulate neural differentiation and, at the same time, to permit differentiation of ES cells to cardiac fate.

Recent studies have investigated the mechanisms underlying neural specification in uncommitted ES cells (Tropepe et al., 2001). However, the role of the pathways that have been implicated in neural generation in the context of stem cells is still under investigation (Munoz-Sanjuan and Brivanlou, 2002). Although a better understanding of the molecu-

lar events that mediate the acquisition of neural fates of ES cells in the absence of Cripto signaling is required, our controlled differentiation paradigm could represent an attractive system to further investigate this issue.

Cripto signaling pathway in cardiomyogenesis

Both genetic and biochemical data indicate a role for Cripto and, more generally, for the EGF-CFC factors in Nodal signaling (Gritsman et al., 1999; Reissmann et al., 2001; Schiffer et al., 2001; Yan et al., 2002). More recently, findings in *Xenopus* and zebrafish have also shown that the TGFβ Vg1/GDF1-like signals depend on EGF-CFC proteins (Cheng et al., 2003).

We observed a significant increase in Smad2 phosphorylation consequent to treatment of Cripto$^{-/-}$ ES cells with recombinant Cripto protein for different lengths of time. This suggests that Cripto signaling acts through the Smad2 pathway to promote cardiac induction and reveals a potential role of Nodal signaling in cardiogenesis. Acute stimulation by Cripto, although competent in activating Smad2, is insufficient to achieve proper terminal cardiac differentiation, again highlighting the importance of signal duration for cardiomyogenesis.

Activation of Alk4/Tar-A signaling and cardiac induction

One critical function of Cripto during development is to render Activin type I receptor competent for activation by Nodal and to potentiate Nodal-triggered Alk7 signaling activity (Gritsman et al., 1999; Reissmann et al., 2001; Yeo and Whitman, 2001; Yan et al., 2002). Here we show that activated forms of either Alk4 or zebrafish Taram-A partially compensate for the lack of Cripto in the cardiomyocyte differentiation assay, suggesting that, indeed, this receptor family is involved in Cripto-mediated cardiomyogenesis. The incomplete rescue could be due to different reasons. As overexpression experiments do not allow the modulation of receptor signaling both in terms of timing and signal strength, we measured the effect of constitutive, but not transient (acute), activation of Alk4-mediated signaling. Cripto may also interact either with other Alk or TGFβ receptor family members or still unknown molecules to promote cardiomyocyte induction and differentiation in ES cells.

Mutational dissection of Cripto

The mutational dissection of Cripto enabled us to define that both the EGF and the CFC domains are crucial for Cripto activity in cardiogenesis. Remarkably, the biological activities of the Cripto mutants in cardiogenic induction correlate well with their effects on Alk4/Nodal signaling. First, the two amino acids located in the EGF domain whose mutation significantly reduces or completely abolishes Cripto activity, namely G71 and F78, also appeared to be strictly required to rescue cell competence to respond to Nodal signaling in the zebrafish assay (Minchiotti et al., 2001). Interestingly, the impaired activity of mutant Cripto protein was dependent on the amino acids chosen for the substitution. In fact, while substitution of phenylalanine to alanine (F78A) significantly reduced protein activity, a tryptophan in the same position (F78W) preserved Cripto ability to promote cardiogenesis. Worth noting, F78 is fully exposed in the 3D

model of Cripto and has been hypothesized to be involved in protein binding (Lohmeyer et al., 1997; Minchiotti et al., 2001). Second, receptor reconstitution experiments in *Xenopus* have indicated that the EGF domain of Cripto is crucial for Nodal binding to the Alk4/ActRIIB receptor complex (Yeo and Whitman, 2001), while the CFC domain was required for Cripto to interact with the Alk4 receptor. Specifically, either double or triple mutations in the CFC domain, including the amino acid W107, have been reported to impair Alk4-dependent Cripto activity (Yeo and Whitman, 2001; Yan et al., 2002). Here, we show that the single amino acid substitution of residue W107 in the CFC domain severely impairs the ability of Cripto to promote cardiac induction in Cripto$^{-/-}$ ES cells. Finally, several reports have described the modification of Cripto by the addition of sugar residues, including a rare case of fucosylation, suggesting that the activity of Cripto may be controlled by the extent of its glycosylation or fucosylation (for review see Rosa, 2002). Here we show that an alanine substitution in the site of O-fucosylation (T72A; Yan et al., 2002) generates a Cripto mutant protein that is still competent to promote cardiomyocyte differentiation, although showing a reduced activity compared with the wt. Although T72A modification of Cripto has been previously shown to be completely inactive in facilitating Nodal signaling in *Xenopus* (Schiffer et al., 2001) and in coculture assay (Yan et al., 2002), recent data showed that mutant embryos lacking O-fucosyltransferase do not resemble the *cripto* knockout phenotype, thus suggesting a less stringent requirement for O-fucose on Cripto activity in vivo than in reporter assay (Shi and Stanley, 2003).

Nodal signaling is required for Cripto-regulated cardiomyogenesis

Results reported herein suggested that Nodal signaling was required for Cripto-regulated cardiac induction and differentiation. To obtain more direct evidence to support this hypothesis, we performed loss-of-function experiments by using Nodal antagonists in our controlled differentiation assay. To this end, either Cerberus or Cerberus-S proteins were used, either by transfecting Cripto$^{-/-}$ ES cells with corresponding expression vectors or by using conditioned media containing the recombinant proteins. In both cases, the presence of either Cerberus or Cerberus-S results in a strong inhibition of Cripto activity in the differentiation assay, thus supporting the idea that Nodal is indeed required to mediate Cripto-dependent cardiomyocyte induction and differentiation of ES cells.

Understanding the early events of lineage segregation during differentiation of mammalian cells is crucial for the prospects of controlling stem cell differentiation for biomedical application. Although ES cells represent a viable source of donor cells for transplantation and gene delivery, the successful use of ES-derived donor cells would require the generation of essentially pure cultures of specific cell types (Boheler et al., 2002). In this respect, our results open new insights into the understanding of the molecular mechanisms by which *cripto* regulates cardiogenesis, and will hopefully contribute to the characterization of the molecular signals that control both cardiac and neuronal differentiation of stem cells as the first step in the ongoing efforts to employ these cells in regenerative medicine.

Materials and methods

Plasmids and mutants

The pallino βA vector was derived from the pallino vector (provided by S. Chiocca, European Institute of Oncology, Milan, Italy) by replacing the CMV promoter with the chicken β-actin promoter (pCXN2 vector; Niwa et al., 1991). Restriction sites were blunt ended using Klenow polymerase. All the *cripto* mutant derivatives were obtained as previously described (Minchiotti et al., 2001). The Cripto-His here renamed "secreted Cripto" and the EGF-CFC derivatives have been previously described (Minchiotti et al., 2001). The *cripto* EGF long (nucleotide −5 to +288 of *cripto* cDNA; Dono et al., 1993), *cripto* EGF short (nucleotide −5 to +75 fused to +157/+288 of *cripto* cDNA), wt and activated (ca) Alk4, wt and activated (ca) Taram-A, Cerberus, and Cerberus-S cDNAs were all subcloned into pallino βA vector.

Cell cultures and ES differentiation

Human embryonic kidney 293 and 293EBNA cells and undifferentiated ES cells were cultured as previously described (Xu et al., 1999; Minchiotti et al., 2001). For in vitro differentiation, ES cells were cultivated in EBs essentially as previously described (Wobus et al., 1991; Maltsev et al., 1993; Fig. 1). The EBs were plated separately onto gelatin-coated 48-well plates for morphological analysis or onto 100-mm tissue culture plates for RT-PCR and Western blot.

Cell transfections and proteins

Undifferentiated ES cells (10^7/ml) were electroporated with linearized DNA (30 µg) at 400 V, 250 µF. 1 wk after selection with 2 µg/ml puromycin, resistant clones were pooled, expanded, and subjected to the differentiation assay. Transfection of 293EBNA cells was performed as previously described (Minchiotti et al., 2000).

Recombinant secreted Cripto proteins were obtained and purified as previously described (Minchiotti et al., 2001). Conditioned media containing either Cerberus or Cerberus-S were obtained from 293T cells as previously described (Piccolo et al., 1999).

Western blotting

Either undifferentiated ES cells or EBs were lysed either in a buffer containing 10 mM Tris/Cl, pH 8, 140 mM NaCl, 2 mM EDTA, pH 8, 1% NP-40 or dissolved in Laemmli lysis buffer (Laemmli, 1970) and analyzed by Western blot using the Trans-Blot Semi-dry System (Bio-Rad Laboratories). The anti-HA (12CA5) antibody (ROCHE), anti-Porin 31HL antibody (Calbiochem), anti-Smad2/3, and anti–phospho-Smad2 (Ser465/467) (Upstate Biotechnology) were used according to the manufacturer's instructions.

RNA preparation and RT-PCR

Total RNA from either undifferentiated ES cells or EBs was extracted with TRIzol kit (Life Technologies) according to the manufacturer's instructions and reverse transcribed to cDNA with SuperScript II reverse transcriptase (Life Technologies) and random hexamers (as primers). cDNA samples synthesized from 100 ng of total RNA were subjected to PCR amplification with specific primers. The primers and the PCR conditions used were as follows: Nodal, 5′-TTCCTTCTCAGGTCACGTTTGC-3′ (forward) and 5′-GGTGGGGTTGGTATCGTTTCA-3′ (reverse), annealing temperature 58°C, cycles 35, producing a 518-bp fragment; ALK-4, 5′-AAGGATCCAG-GCTCTGCTGTGTGCC-3′ (forward) and 5′-ACGGATCCATGTCCAAC-CTCTGGCGG-3′ (reverse), annealing temperature 60°C, cycles 30, 411-bp fragment; ActRIIB, 5′-ATGTGCCGTGGTGTCGTGGT-3′ (forward) and 5′-GACCTCCTGATCAGGGATAC-3′ (reverse), annealing temperature 58°C, cycles 30, 54-bp fragment; MLC2v, 5′-GCCAAGAAGCGGATAGAAG-GCGGG-3′ (forward) and 5′-CTGTGGTTCAGGGCTCAGTCCTTC-3′ (reverse), annealing temperature 70°C, cycles 33, 490-bp fragment; cardiac αMHC, 5′-GGAAGAGTGAGCGGCGCATCAAGG-3′ (forward) and 5′-CTG-CTGGAGAGGTTATTCCTCG-3′ (reverse), annealing temperature 65°C, cycles 30, 301-bp fragment. A set of primers for hypoxanthine phosphoribosyltransferase (HPRT), 5′-CCTGCTGGATTACATTAAAGCACTG-3′ (forward) and 5′-CCTGAAGTACTCATTATAGTCAAGG-3′ (reverse), annealing temperature 58°C, cycles 25, 369-bp fragment, was used as a control.

Immunofluorescence of EBs

Adherent EBs were fixed either in methanol/acetone (7:3; MF-20 antibody) or in 4% paraformaldehyde in PBS (β-tubulin isotype III). EBs were treated with 0.1% Triton X-100 (Sigma-Aldrich), 10% normal goat serum (DakoCytomation) in PBS and incubated with primary antibodies in 10% NGS, 1× PBS at the following working dilutions: anti–neurofilament-M (1:400; Chemicon International), anti–β-tubulin isotype III (1:400; Sigma-Aldrich),

and anti–sarcomeric myosin (MF-20, 1:50; monoclonal supernatant obtained from the Developmental Studies Hybridoma Bank, University of Iowa). After washing, EBs were incubated with secondary antibodies, either fluorescein (Boehringer) or rhodamine conjugated (Jackson ImmunoResearch Laboratories), in 10% NGS, 1× PBS. After PBS wash, EBs were counterstained with DAPI and mounted in Vecta Shield medium (Vector Laboratories). Labeling was visualized by epifluorescent illumination using an Axioskop 2 microscope, and images were acquired on an Axiocam ARC camera (Carl Zeiss MicroImaging, Inc.).

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