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Regulation of p57^{KIP2} during Muscle Differentiation: Role of Egr1, Sp1 and DNA Hypomethylation

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The cdk inhibitor p57^{kip2} plays a critical role in many differentiation processes by performing not only redundant but also specific functions. Compared to other cdk inhibitors, p57^{kip2} shows a more restricted expression pattern during development and in adult tissues.

We have previously reported that in muscle cells, p57^{kip2} is induced by the myogenic factor MyoD through an indirect mechanism involving p73 proteins as intermediaries. We have also reported that p57^{kip2} shows a differential responsiveness to MyoD-dependent regulation in different cell types.

In this work we have further investigated the molecular mechanism by which MyoD activates p57 promoter. We show that the minimal promoter element able to confer MyoD responsiveness contains multiple Sp1 and Egr1 recognition sites and that both transcription factors are necessary for the increase in p57 RNA. We also suggest that the role of MyoD-induced p73 consists in promoting the binding of Sp1 to p57^{kip2} promoter. Moreover, we show that Egr1 and Sp1 are concomitantly recruited to p57 promoter *in vivo* only in differentiation conditions and only in responsive cells. Bisulfite sequencing suggested a functional link between the methylation status and the differential activity of p57 promoter, both during differentiation and in distinct cell types. These results, which highlight the involvement of epigenetic factors in the regulation of p57 expression in muscle cells, could be of general relevance to explain its tissue and cell type restriction during development.

Keywords: cdk inhibitors; muscle differentiation; p57^{kip2}; promoter methylation

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Introduction

Cyclin-dependent kinase inhibitors (CKIs) are fundamental regulators of development in multicellular organisms.[1] The best established role of CKIs consists in promoting and maintaining cell cycle arrest in terminally differentiating and quiescent cells. However, their function is not completely redundant, as inferred by the different phenotypes resulting from single CKI knockouts in mouse models.[2] The expression patterns of CKIs are only in part overlapping, thus suggesting their involvement in different developmental pathways. Furthermore, the members of the Cip/Kip family—namely, p21$^{Cip1/Waf1}$ (p21), p27^{Kip1} (p27) and p57^{Kip2} (p57)—have been shown to play specific additional roles besides cdk inhibition.[3] These findings suggest that CKIs are under the control of different regulatory pathways in order to exert distinct functions in different cell types.

The function and the regulation of p57 are particularly appealing. p57 is the most structurally complex member of the Cip/Kip family and the only CKI whose individual ablation causes developmental defects and neonatal lethality in mice.[4,5]

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Abbreviations used: CKI, cyclin-dependent kinase inhibitor; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid.

Moreover, the gene coding for p57 is paternally imprinted both in humans and in mice and is located, together with insulin-like growth factor-II, within an imprinted cluster involved in growth regulation and development and associated with the Beckwith–Wiedemann syndrome.[6]

In contrast to the widespread expression of p21[7] and p27,[8] p57 shows a more restricted tissue distribution. Although a detailed analysis has not been reported, the general picture is that p57 is predominantly expressed during embryogenesis. However, p57 RNA and/or protein have been detected in some adult tissues and organs, such as placenta, muscle, heart, brain, lung and kidney.[9,10]

In skeletal muscle, p57 cooperates with p21 in supporting terminal differentiation. While neither of the single knockout mice shows overall muscle defects, apart from a body wall muscle dysplasia in the p57 knockout, double mutants are severely impaired in myogenesis due to hyperproliferation and apoptosis of myoblast cells.[11] However, it is not clear whether the two CKIs act in a purely redundant manner or their cooperation involves compensatory mechanisms, such as the assumption of a biochemical function usually not performed or an adjustment of their spatial and/or temporal expression pattern. Consistent with its role in myogenesis, p57 expression is up-regulated during muscle regeneration following injury[12] and in myoblasts differentiating *in vitro*.[13,14] Interestingly, unlike that observed for p21, the expression of p57 is restricted to only some experimental systems of *in vitro* myogenesis[14,15] and is detectable only in a subset of the myotube nuclei *in vivo*.[4] This suggests that p57 can be induced in a subpopulation of muscle precursors, probably in order to exert specific functions. A related phenomenon has been observed during neurogenesis, in particular in developing retina and spinal cord, in which different Cip/Kip inhibitors are expressed in distinct progenitor cell types.[16,17]

Despite the clear importance of p57 in development, little is known about the mechanisms regulating its expression during differentiation processes. We have recently reported that during muscle differentiation, p57 is induced at the transcriptional level by the muscle regulatory factor MyoD through a mechanism different from that involved in the up-regulation of p21.[14,15] We identified a p57 promoter region as sufficient to confer MyoD responsiveness in reporter assays. However, while p21 is directly induced by MyoD in the absence of new synthesised factors, the up-regulation of p57 requires p73 family members as intermediaries. The trans-activation mechanisms appeared to be quite complex. In fact, we found that the minimal p57 promoter element does not contain canonical p73 binding sites, suggesting that additional factors mediate DNA recognition. Moreover, we observed that transfected p57 promoter–reporter constructs were responsive to MyoD-induced p73 even in cell types unable to express p57, suggesting that some cis-acting mechanism may repress the endogenous promoter in certain cell contexts. In this work, taking advantage of differentially responsive cell types, we have further analyzed the mechanisms underlying the induction and restriction of p57 expression in muscle cells. We show that the induction of p57 by MyoD requires, besides p73, also Egr1 and Sp1 transcription factors and that p73 participates in the Sp1-containing complex bound to the p57 regulatory region. Moreover, we suggest that the activation of p57 promoter requires not only the induction of trans-acting factors, but also the removal of an epigenetic constraint involving DNA methylation.

Results

Differential expression of p57 in various myogenic cell types

Differentiating myoblasts express both p21 and p57. We have recently reported that the expression of the two cdk inhibitors is induced by the myogenic factor MyoD through different mechanisms.[15] By expressing exogenous MyoD in fibroblasts, a common approach to analyzing MyoD activities, we have also reported that different fibroblast cell types, equally competent to undergo myogenic conversion, are differentially responsive with regard to the induction of p57.[14] In particular, fibroblast cells from p21 knockout mice are highly responsive, while other fibroblast cell lines, such as Balb C-3T3, Swiss-3T3 and Swiss-3T6, are completely refractory. It is improbable that the sensitivity of p57 to MyoD-dependent regulation has been just selected in $p21^{-/-}$ fibroblasts, since we have observed that primary mouse embryo fibroblasts, wild type for p21, are capable of up-regulating p57 upon MyoD expression (A. Busanello, unpublished observation). In this work, we used $p21^{-/-}$ spontaneously immortalized fibroblasts as a reliable and handy experimental model of responsive cells. To better determine the expression levels and to compare the cellular distribution of the two CKIs, we performed double-immunofluorescence staining for p21 and p57 in MyoD-converted fibroblasts and in differentiating C2.7 myoblasts. To express exogenous MyoD, $p21^{-/-}$ (responsive) and C3H10T1/2 (unresponsive) fibroblasts were infected with a high-titer MyoD retroviral vector,[18] enabling homogeneous and high-level expression in transient assays. Moreover, in the case of $p21^{-/-}$ fibroblasts, a co-infection with both p21 and MyoD retroviral vectors was performed in order to determine the possible effects of p21 on the induction of p57 in the same responsive cell background. Infected fibroblasts and C2.7 myoblasts grown to confluence were shifted to differentiation medium and, 48 h later, were fixed and immunostained. As shown in Fig. 1, this analysis confirms the reverse behaviour of the two fibroblast cell types regarding the induction of either of the two CKIs. However, it also reveals that in both C2 myoblasts and p21/MyoD-infected fibroblasts, p21 and p57 are frequently co-localized in the same nucleus, thus