Review

Arresting developments in the cardiac myocyte cell cycle: Role of cyclin-dependent kinase inhibitors

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Abstract

Like most other cells in the body, foetal and neonatal cardiac myocytes are able to divide and proliferate. However, the ability of these cells to undergo cell division decreases progressively during development such that adult myocytes are unable to divide. A major problem arising from this inability of adult cardiac myocytes to proliferate is that the mature heart is unable to regenerate new myocardial tissue following severe injury, e.g. infarction, which can lead to compromised cardiac pump function and even death. Studies in proliferating cells have identified a group of genes and proteins that controls cell division. These proteins include cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs), which interact with each other to form complexes that are essential for controlling normal cell cycle progression. A variety of other proteins, e.g. the retinoblastoma protein (pRb) and members of the E2F family of transcription factors, also can interact with, and modulate the activities of, these complexes. Despite the major role that these proteins play in other cell types, little was known until recently about their existence and activities in immature (proliferating) or mature (non-proliferating) cardiac myocytes. The reason(s) why cardiac myocytes lose their ability to divide during development remains unknown, but if strategies were developed to understand the mechanisms underlying cardiac myocyte growth, it could open up new avenues for the treatment of cardiovascular disease. In this article, we shall review the function of the cell cycle machinery and outline some of our recent findings pertaining to the involvement of the cell cycle in modulating cardiac myocyte growth and hypertrophy. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cell cycle; CIP/KIP; Cyclin; Cyclin-dependent Kinase inhibitor; Heart; Myocyte

1. Introduction

1.1. Developmental regulation of cardiac myocyte growth

During the early stages of mammalian development, cardiac myocytes grow both by hyperplasia (cell division, where the entire cell contents are duplicated, followed by the cell dividing into two daughter cells) and hypertrophy (an increase in cell size without a concomitant increase in cell number), resulting in an overall increase in the mass of the heart [1]. Myocyte proliferation ceases completely by about 17 days post-natal development in the rat, with all subsequent increases in heart size being due solely to hypertrophy [1], although it has been reported recently that the switch from hyperplastic to hypertrophic growth in the rat heart occurs between days three and four of post-natal development [2]. Furthermore, in the mouse, all cardiac myocyte cell division has ceased by birth, although DNA synthesis continues in neonatal cells, which contributes to myocyte binucleation [3]. The inability of adult cardiac myocytes to proliferate has been attributed to a block in

Abbreviations: CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; pRb, retinoblastoma protein; LVH, left ventricular hypertrophy; R point, restriction point; TGF-β, transforming growth factor-β; MTS, multiple tumour suppressor; AC, aortic constriction; SH, sham; LV, left ventricular; FACS, fluorescence-activated cell sorting; mRNA, messenger ribonucleic acid; PCNA, proliferating cell nuclear antigen

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the cell cycle, which occurs shortly after birth [4]. Thus, following severe injury, such as occurs during myocardial infarction, the mature heart is unable to regenerate new myocytes to replace necrotic or damaged tissue. Despite the diminishing ability of the cardiac myocyte to undergo cell division, it is able to synthesise DNA throughout its life, if required, which is thought to reflect either the formation of binucleated cardiomyocytes and/or an increase in the ploidy of the myocyte nuclei, rather than an increase in cell number, which would require myocyte cell division [5]. In the adult rat heart, there appears to be a block in the cell cycle, with the majority of myocytes being locked in either the G₀ or G₁ phases of the cell cycle [4], consistent with terminal differentiation of these cells. However, our recent studies using fluorescence-activated cell sorting (FACS) analysis of propidium iodide- and bromodeoxyuridine-labelled myocyte nuclei have suggested that an additional, and significant, block occurs also at the G₂/M phase of the cell cycle. Thus, whereas 85% of adult myocytes were found in G₀/G₁, 15% were found in the G₂/M phases (Poolman et al., submitted). In accordance with our observation of a significant G₂/M block in cardiac myocytes, Rumyantsev [6] has suggested that, even during the prenatal stages of rat and mouse cardiomyogenesis, a significant number of myocytes remain in G₂, thereby forming a population of G₂-arrested cells.

In additional studies, Anversa et al. [7] and Olivetti et al. [8] have shown that, in some instances, cardiac myocyte nuclear division can be re-initiated in the adult rat heart. However, this re-initiation of myocyte nuclear division appears to occur only in decompensated hearts that are aged or have been subjected to extreme, long term stress and there is no evidence that this phenomenon occurs in normal adult hearts.

1.2. Left ventricular hypertrophy (LVH): A major risk factor for cardiovascular morbidity and mortality

LVH now is recognised widely as a powerful cardiovascular risk factor. Data from the Framingham study have shown that LVH is associated with all manifestations of ischaemic heart disease (e.g. myocardial infarction and cardiac failure), and that there is a particularly strong association with an increased incidence of sudden cardiac death [9]. Except in the case of cardiomyopathies, cardiac hypertrophy generally is an adaptive, physiological response of the heart to stress, imposed as a result of e.g. hypertension or myocardial infarction [10]. It is important to note, however, that the hypertrophic response produced by left ventricular overload is not in itself a pathological state, since, as a result of normal development, the heart undergoes this process. A number of investigators have shown that cardiac hypertrophy is associated with qualitative and quantitative changes in the expression of various genes, in many cases, towards the foetal phenotype (see [11] for review). These include genes encoding for atrial natriuretic factor [12], sarcolemmal ion transporting proteins, such as Na⁺/K⁺ ATPase α subunits [13] and the Na⁺/Ca²⁺ exchanger [11,14], and structural proteins, such as β-myosin heavy chain (β-MHC) and skeletal muscle α-actin [15,12], changes in expression of which are the reverse of those observed during post-natal development [11,16–18]. However, little is known about those molecules that act upstream and induce these changes in gene expression during hypertrophic growth, although recent findings from our laboratory have demonstrated that cell cycle-dependent molecules may play an important role (19 and see below).

2. The mammalian cell cycle

The cell cycle is divided into five distinct phases (see Fig. 1). Quiescent cells are found in the G₀ phase of the cycle and exist in a state where mRNA and protein synthesis are minimal. Cells may remain in this state for many years but can re-enter the cell cycle, within G₁, when required. During the G₁ phase, or the first gap phase, the cell synthesises mRNAs and proteins in readiness for DNA synthesis, which occurs during the S phase of the cycle. Following DNA synthesis, the cell enters a second gap phase known as G₂, when the cell synthesises additional mRNAs and proteins in preparation for cell division or mitosis, which occurs during the M phase of the cycle. Cell cycle progression is known to be under the control of cell cycle-dependent molecules and is regulated both positively, through the formation and activation of various cyclin and cyclin-dependent kinase (CDK) complexes, and negatively, by CDK inhibitors (CDKIs; see [20] for review). A variety of other proteins, e.g. retinoblastoma protein (pRb), proliferating cell nuclear antigen (PCNA) and members of the E2F transcription factor family, also can interact with, and modulate the activity of, these complexes.

A number of cell cycle checkpoints exist within the cell that are essential for healthy cell division. The primary cell cycle checkpoint coordinating cell growth and proliferation in mammalian cells is called the restriction (R) point and once a cell has passed this point, it is committed to a further round of DNA replication (S phase) and cell division (mitosis), except in fully differentiated cells such as adult cardiac myocytes, where DNA synthesis can occur in the absence of cell division.

We previously have reviewed the area of cyclins and CDKs in cardiac myocyte development in detail [20] and, therefore, will limit this review to a discussion of those CDKIs that are expressed in cardiac myocytes and their potential role(s) in mediating cell cycle arrest of developing cardiac myocytes and in hypertrophic growth.
3. The cyclin-dependent kinase inhibitors (CDKIs)

The CDKIs are a recently described class of cell cycle regulatory molecules that exert a negative effect on the cell cycle machinery by binding to, and inhibiting the activities of, various cyclin–CDK complexes. Currently, two structurally defined classes of CDKIs exist in mammals: (i) the INK4 family, which includes p14 [21], p15\textsuperscript{INK4B} [22], p16\textsuperscript{INK4A} [23,24], p18\textsuperscript{INK4C} [21,25] and p19\textsuperscript{INK4D} [25], and (ii) the KIP/CIP family, which includes p21\textsuperscript{CIP1} [26,27], p27\textsuperscript{KIP1} [28,29] and p57\textsuperscript{KIP2} [30,31]. Members of the KIP/CIP family are structurally unrelated to the INK4 family and, in vitro, the KIP/CIP family shows a broader specificity, since it interacts with, and inhibits the kinase activities of, cyclin E–CDK2, cyclin D–CDK4, cyclin D–CDK6 and cyclin A–CDK2 complexes (Table 1).

Table 1
Properties of the KIP/CIP and INK4 CDKIs

<table>
<thead>
<tr>
<th>Property</th>
<th>KIP/CIP family (p21, p27, p57)</th>
<th>INK4 family (p14, p15, p16, p18, p19)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK binding site</td>
<td>N terminus</td>
<td></td>
<td>[29–31,34,37,40]</td>
</tr>
<tr>
<td>Amino acid sequence homology</td>
<td>Little between families.</td>
<td>High within each family, especially at the N-terminus (CDK binding site)</td>
<td>[21,22,25,30,31,34] [37,40]</td>
</tr>
<tr>
<td>Nuclear localisation signal</td>
<td>C terminal</td>
<td>No signal found</td>
<td>[30,34]</td>
</tr>
<tr>
<td>Ankyrin repeats</td>
<td>Not found</td>
<td>Series of four ankyrin repeats (32 amino acids long). Involved in protein–protein interactions</td>
<td>[22,23,25,35]</td>
</tr>
<tr>
<td>Mutated or deleted in cancer cells</td>
<td>Rarely</td>
<td>Frequently (especially p16)</td>
<td>[24,56,74,75,104–106]</td>
</tr>
<tr>
<td>Overexpression</td>
<td>Causes G\textsubscript{1} phase cell cycle arrest</td>
<td></td>
<td>[22,30,31,34,37,41,42] [79,102]</td>
</tr>
</tbody>
</table>
[26,29–34] compared with the INK4 CDKIs that inhibit CDK4 and CDK6 complexes only (Table 1). Furthermore, the aligned amino acid sequence identities have been determined previously for both the INK4 [21,22,25,35] and the KIP/CIP [23,30,31,34,36–40] CDKIs and have revealed little sequence homology between the two families. However, within each family there is high sequence homology, especially at the amino terminus, which contains the CDK binding site. For example, within the KIP/CIP family, p27<sup>KIP1</sup> shows 44 and 47% identity to p21<sup>CIP1</sup> and p57<sup>KIP2</sup>, respectively, whereas p21<sup>CIP1</sup> is 36% identical to p57<sup>KIP2</sup> within the N-terminal portion of each protein [30,34]. Primarily the CDKIs cause cell cycle arrest in the G<sub>i</sub> phase of the cell cycle [41,42] and this is consistent with the proposed block in the cell cycle seen in rat cardiac myocytes beyond 17 days of post-natal development [4]. The nomenclature of the CDKIs is complex, due to identification of these molecules by several independent groups, but, for clarity, we shall refer to the inhibitors as p14, p15, p16, p18, p19, p21, p27 and p57 (representing the molecular weights of the proteins as measured by polyacrylamide gel electrophoresis). The two families of CDKI molecules display different properties and targets, which are summarised in Tables 1 and 2 below. Interestingly, no INK4 family members were detected at the protein level in cardiac myocytes at any stage of development (Poolman and Brooks, unpublished observations); therefore, in this review, we will limit our discussion to those members of the KIP/CIP family that are expressed in cardiac myocytes. Further details of the INK4 family members can be found in several excellent reviews (e.g. [43]).

### 3.1. p21

The first member of the KIP/CIP family to be identified and cloned was the heat-stable protein, p21 [26,32,36,44–47]. This CDKI appears to be a universal inhibitor of

<table>
<thead>
<tr>
<th>CDKI molecule</th>
<th>Inhibitory target</th>
<th>Regulation and effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p14</td>
<td>CDK4 and CDK6</td>
<td>Unknown</td>
<td>[21]</td>
</tr>
<tr>
<td>p15</td>
<td>CDK4</td>
<td>TGF-β induces mRNA.</td>
<td>[22,104,107]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low protein expression in proliferating cells</td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>CDK4</td>
<td>Regulation unknown.</td>
<td>[22,41,100,102,108]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Competes with cyclin D for binding to CDK4/6.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppression of cell growth requires functional pRb.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcription repressed by pRb.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits cyclin D&lt;sub&gt;i&lt;/sub&gt; activation of the E2F promoter</td>
<td></td>
</tr>
<tr>
<td>p18</td>
<td>CDK4 (weak binding)</td>
<td>Suppression of cell growth</td>
<td>[25,109]</td>
</tr>
<tr>
<td></td>
<td>CDK6 (strong binding)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p19</td>
<td>CDK4 and CDK6</td>
<td>Suppression of cell growth</td>
<td>[25,35]</td>
</tr>
<tr>
<td>p21</td>
<td>Cyclin A–CDK2</td>
<td>p53-dependent</td>
<td>[26,28,38,54,59,66]</td>
</tr>
<tr>
<td></td>
<td>Cyclin E–CDK2</td>
<td>γ-radiation induces mRNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin D–CDK2/4/5/6</td>
<td>p53-independent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin A/B–CDC2</td>
<td>Mitogenic (i.e. PDGF) or differentiation factors induce transcription (e.g. MyoD in skeletal muscle).</td>
<td>[67,70,79,95,110–112]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCNA-dependent DNA replication</td>
<td></td>
</tr>
<tr>
<td>p27</td>
<td>Cyclin E–CDK2</td>
<td>Constant mRNA expression throughout cell cycle.</td>
<td>[28,29,34,79–85]</td>
</tr>
<tr>
<td></td>
<td>Cyclin A–CDK2</td>
<td>Protein expression peaks in G&lt;sub&gt;i&lt;/sub&gt;.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin D–CDK4</td>
<td>Degraded by the ubiquitin–proteasome pathway.</td>
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<tr>
<td></td>
<td></td>
<td>Sequestered by a heat-labile complex</td>
<td></td>
</tr>
<tr>
<td>p57</td>
<td>Cyclin E–CDK2</td>
<td>Highly expressed in terminally differentiated cells.</td>
<td>[30,31]</td>
</tr>
<tr>
<td></td>
<td>Cyclin A–CDK2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin D–CDK4</td>
<td>Regulation unknown</td>
<td></td>
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<td>Regulation unknown.</td>
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<td></td>
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</tr>
<tr>
<td>p19</td>
<td>CDK4 and CDK6</td>
<td>Suppression of cell growth</td>
<td>[25,35]</td>
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<td></td>
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<td></td>
<td>Cyclin D–CDK4</td>
<td>Regulation unknown</td>
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</table>
cyclicity activity [26,33,44], primarily acting in G1 and at the G1–S phase transition (Fig. 1). As with all CDKI molecules, the cyclin–CDK binding sites are found at the N-terminus of the p21 molecule [37,48,49], although a second weaker cyclin-binding motif has been discovered recently in the C-terminal region of p21 [50]. In normal cells, p21 exists primarily in a quaternary complex with a cyclin, a CDK and PCNA [33,38,51]. PCNA is a nuclear protein that is essential for DNA replication and repair by DNA polymerase 6 and it has been shown that the C-terminal domain of p21 can directly inhibit DNA replication, but not repair, by binding to and inhibiting PCNA activity [48,49,52]. Thus, p21 can exist in both active and inactive cyclin–CDK complexes and it has been suggested that multiple p21 subunits are required to inhibit cyclin

CDK activity [53]. In support of this hypothesis, Zhang et al. [53] have shown recently that p21 exists both in catalytically active and inactive cyclin–CDK complexes and that the addition of subsaturating concentrations of p21 to cyclin A–CDK2 complexes resulted in a progressive increase in CDK2 activity, suggesting that low concentrations of p21 might function as a cyclin–CDK assembly factor, whereas the binding of more than one p21 molecule is apparently required to inhibit CDK2 activity [53]. The association of p21 with PCNA occurs only in non-transformed cells, since the quaternary complexes are disrupted, and p21 and PCNA are no longer bound, in transformed cells [33,38,51].

The level of p21 mRNA has been shown to fluctuate during the cell cycle, peaking on exit from the G1 phase [36,38] and at the G1/M transition [38]. It has been proposed that the induction of p21 on exit from G1 is to reduce cyclin–CDK activity in G1 and to act as a threshold, above which cyclin–CDK complexes need to accumulate to enable cell cycle progression [38]. In support of this hypothesis, the levels of p21 mRNA and protein are elevated in quiescent and differentiated cells [38,54–62] and overexpression of p21 caused either a cell cycle delay or prevented cellular proliferation [33,37,44,63–65]. Furthermore, expression of antisense p21 RNA in G1-arrested HCA2 human diploid fibroblasts resulted in the induction of DNA synthesis as well as entry into mitosis [39]. Taken together, these results suggest that decreased expression of the p21 gene is necessary for cell cycle progression. Initial studies suggested that regulation of p21 expression was dependent solely upon the tumour suppressor, p53, which induces transcription of the p21 gene, thereby causing cell cycle arrest and growth suppression [33,52,55,66–68]. However, subsequent studies have demonstrated that p21 can be activated also in a p53-independent manner in response to multiple mitogenic stimuli [e.g. platelet-derived growth factor, epidermal growth factor, fibroblast growth factor and transforming growth factor-β (TGF-β) [55,69,70]] and differentiation-inducing factors (e.g. okadaic acid, vitamin D3, retinoic acid, dimethyl sulphoxide and phorbol 12-myristate 13-acetate) that often result in G1 arrest [54,60,71–73]. Thus, p21 appears to function via two opposing mechanisms: (i) as a CDKI (p53-dependent) and (ii) as an immediate early gene that is induced in response to mitogens and differentiation stimuli (p53-independent). Given the multiple roles and universal effects of p21 in the cell, it was expected that the deletion of the p21 gene would result in disastrous consequences. However, in comparison to p27 knockout mice (see Section 3.2), p21 knockout mice developed quite normally and showed no spontaneous malignancies during seven months of post-natal observation [74]. The lack of tumour formation in these mice was consistent with the failure to detect p21 gene mutations in a variety of human tumours [75]. The results of these p21 knockout mouse studies suggest that the role of p21 in terminal differentiation and exit of cells from the cell cycle must be redundant in the mouse. However, a potential role for p21 in cardiac myocyte exit of cells from the cell cycle progression. Initial studies suggested that regulation proliferation [28,54,79–84]. The abundance of p27 protein was first discovered in TGF-

b

b

2

mice were shown

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body was detected, including the heart [86–88]. This organ enlargement was shown to be due to an increase in cell number, rather than an increase in cell size, thereby making this model an ideal system for determining the effects of this CDKI on the cardiac myocyte cell cycle. Studies currently are underway in our laboratory to investigate this further.

3.3. p57

The most recently described KIP/CIP CDKI protein is p57 [30,31]. Mouse p57 possesses four structurally distinct domains; an amino terminal CDK inhibitory domain, a proline-rich domain, an acidic domain and a carboxy terminal domain, which contains a putative nuclear localisation signal and a CDK consensus phosphorylation site [30]. It should be noted that this unique domain structure was not conserved in human p57 [31]. p57 has been shown to be a potent inhibitor of G1 and S phase CDK complexes (cyclin D–CDK4 and cyclin E/A–CDK2, respectively; Table 2) but displays less inhibitory activity towards the mitotic cyclin B–CDC2 complex [30,31]. p57 protein localizes to the nucleus and has been shown to be highly expressed in a wide range of human tissues, including skeletal muscle, brain, heart, lungs and eye [30,31]. Whereas overexpression of p57 has been shown to cause G1 arrest [30,31], mice lacking the imprinted p57 gene recently have been shown to possess altered cell proliferation and differentiation, leading to abdominal muscle defects, cleft palate, endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes, renal medullary dysplasia, adrenal cortical hyperplasia and cytomegaly, and lens cell hyperproliferation and apoptosis [89]. Indeed, loss of p57 expression has been proposed to play a pivotal role in Beckwith-Wiedemann syndrome, a pleiotropic hereditary disorder characterised by overgrowth and a predisposition to cancer, since these patients display many of the phenotypic changes observed in p57−/− mice.

4. Expression of CDKI molecules during cardiac myocyte development

As a result of an extensive developmental study using cardiac myocytes obtained from foetal (18 days gestation), neonatal (two-day-old) and adult rats, we were unable to demonstrate expression of any INK4 CDKI family members, despite detection of these molecules in control cell lines (Poolman and Brooks, unpublished observations). In contrast, we were able to demonstrate differential expression of certain KIP/CIP CDKIs during development of the rat myocardium [76]. Thus, in more than six separate experiments, we showed that both whole ventricular tissue and isolated cardiac myocytes displayed a significant upregulation in p21 mRNA and protein expression during development. p27 was expressed at detectable levels in both whole ventricular tissue and in isolated cardiac myocytes, although overall expression of this CDKI in myocytes appeared to be lower than p21 and the extent of upregulation of p27 expression in cardiac myocytes during maturation was shown to be less (~two-fold) than that observed for p21 (~five-fold) (Poolman et al. submitted). In subsequent experiments, we have shown that both p21 and p27 are expressed in the nuclei of cardiac myocytes [19]. Burton et al. [90] have shown recently that p57 protein expression peaks around day 15 of foetal development in the rat and that levels then decline rapidly, such that p57 protein is not detectable after day 19 of foetal development. Lee et al. [30] showed that p57 was expressed at the mRNA level in a tissue-specific manner, such that a 7-kb mRNA species was observed in human skeletal muscle and in heart tissue [30]. However, whether p57 protein was present in these tissues was not documented. In accordance with our findings, Koh and colleagues also have evidence for the developmental regulation of KIP/CIP CDKIs during development of the rat cardiac myocyte (Koh G-Y, personal communication) and Flink et al. [91] have reported that p21 and p27 inhibitory activities increased markedly in cultured neonatal (two-day-old) cardiac myocytes when compared to cultured foetal (16 days gestation) cells [91].

Given that the predominant function of p21 is to inhibit the activity of a plethora of cyclin–CDK complexes [26,33,44], we recently have extended our initial studies and shown that a heat-stable protein lysate containing p21 protein, obtained from adult myocytes, was able to inhibit significantly the histone H1 kinase activity of CDK2 obtained from neonatal rat cardiac myocyte lysates. In addition, we have shown that immunodepletion of p21 from these adult myocyte lysates, prior to incubation with neonatal myocyte lysates, led to a loss of inhibitory activity (Poolman et al., submitted). Therefore, it is feasible that the up-regulation of the KIP/CIP family of CDKI molecules, especially p21, during cardiac myocyte development could contribute to the progressive withdrawal of maturing cardiac myocytes from the cell cycle. Current work in this area is focussing upon the determination of the mechanisms involved in p21 up-regulation in the developing cardiac myocyte.

5. Possible role of CDKIs in mediating cardiac myocyte growth during development

The precise mechanisms that control the cardiac myocyte cell cycle and, therefore, regulate myocyte proliferation and differentiation still remain to be fully understood. However, our recent studies of certain inhibitory elements of the cell cycle (p21 and p27) suggest that certain CDKIs, especially p21, are crucial for controlling
the progressive withdrawal of cardiac myocytes from the cell cycle during development.

It is known that the major function of all CDKI molecules is to inhibit the activity of cyclin–CDK complexes and that the precise way in which this is achieved depends upon whether KIP/CIP or INK4 CDKI molecules are involved (Table 1). Thus, the KIP/CIP family of CDKI molecules inhibit intact cyclin–CDK complexes, whereas INK4 family members prevent the association of cyclins with their respective CDK partners. The block in the cardiac myocyte cell cycle (at three–four days post-natal development in the rat [2]) was previously reported to occur during G1 [4] and, in accordance with this, we have shown that over 85% of adult cardiac myocytes are found in the G0 or G1 phases of the cell cycle [76]. However, our recent studies, using FACS analysis of myocyte nuclei, have suggested that a dual mechanism of myocyte cell cycle arrest exists, with a significant G1/M-phase blockade in adult myocytes [76,92], supporting a hypothesis proposed previously by Rumyantsev [6], suggesting that a population of myocytes begins to arrest during the prenatal stages of cardiac development. Since p21 and p27 were the only two CDKIs detected in myocytes (see Section 4 above) and that these molecules can inhibit the activity of G1 phase CDKs (Fig. 1) and potentially cause a G1-phase cell cycle arrest, it is possible that one or both of these molecules are involved in arresting adult cardiac myocytes. In addition, since p21 mRNA has been shown to peak at the G1/M phase, as well as on exit from G0 [38], and that p21 can inhibit the activity of the G1/M-phase CDK, CDC2, [26,33,44], it is possible that p21 also is critical for arrest of adult myocytes in the G1/M phase of the cell cycle.

Recent observations from our laboratory indicate that, in addition to an upregulation in the expression and inhibitory activities of p21 and p27, there is a concomitant down-regulation in the expression and activities of G1-phase CDKs during development of the rat cardiac myocyte [20,93,94]. Thus, it is conceivable that the up-regulation of the KIP/CIP CDKIs during normal development contributes to the down-regulation of residual G1-phase CDK activity, leading to the arrest of cardiac myocytes in the G1 phase of the cell cycle. However, other potential functions of CDKI molecules exist, especially for p21, which may be involved in the withdrawal of cardiac myocytes from the cell cycle. For example, p21 has been shown to inhibit the activity of PCNA [95], leading to cell cycle arrest at, or just prior to, the S phase. However, we and others have shown that expression of PCNA is down-regulated during development of the cardiac myocyte [5,96] and, therefore, it is unlikely that p21 acts via PCNA to induce a G1 arrest in these cells.

Recently, it has been shown that p21 can suppress the transcriptional activity of E2F-1 [97], a transcription factor that is essential for the induction of several S phase genes, e.g. cyclin A [98]. The suppression of E2F-1 activity results in the loss of induction of S phase genes, with a subsequent block in the cell cycle prior to S phase entry. It is possible, therefore, that, during normal development of the rat cardiac myocyte, p21 may contribute to the progressive loss of myocyte proliferation by inhibiting the transcriptional activity of E2F-1. In support of this hypothesis, we have shown recently that expression of E2F-1 protein is down-regulated significantly during myocyte development such that highest levels are found in foetal and neonatal myocytes, with levels being undetectable in adult cells (Naseem and Brooks, unpublished).

6. Possible role of CDKIs in mediating cardiac myocyte hypertrophy

In addition to determining changes in cell cycle-dependent molecules during normal myocyte development, we also have monitored the expression of these molecules during the development of cardiac hypertrophy. Thus, we have determined p21 and p27 mRNA and protein expression during the development of pressure overload-induced LVH in the rat [19] and shown that there was a significant, but transient, down-regulation in the mRNA and protein expression of these two CDKIs during the 3–14 day post-operative period in LV tissue of rats that had undergone aortic constriction (AC) compared with sham (SH)-operated control animals. Levels of p21 and p27 in aortic constriction (AC) rat hearts subsequently returned to SH levels by 21 days following operation. Immunocytochemical analyses of LV cryosections demonstrated that this down-regulation of p21 and p27 protein levels during the development of LVH occurred in myocyte nuclei specifically [19]. Recently, additional studies have shown that the expression and activities of certain positive regulators of the cell cycle are up-regulated concomitant with the down-regulation of p21 and p27 during the development of LVH [99]. Thus, activities and protein expression of the G1 CDKs, CDK4 and CDK6, were up-regulated by seven days post-operation, with levels returning to those seen in SH-operated controls by six weeks post-operation. Therefore, the cardiac myocyte appears to reinitiate a foetal programme of cell cycle regulatory molecule gene and protein expression during the development of LVH in a similar manner to that reported for certain other genes (see Section 1.2 above).

Interestingly, the changes in expression of both positive and negative regulators of the cell cycle observed during the development of LVH occurred during the adaptive growth phase of the LV following pressure overload, such that the LV weight/body weight ratio plateaued at its maximum value (~50% increase in LV mass) approximately 21 days following AC, corresponding to the period when the changes in expression of cell cycle regulatory molecules had returned to control values. Thus, an alteration in the balance of positive–negative cell cycle regula-
tory molecule expression appears to be crucial for the adaptive growth of the LV (and, specifically, the myocyte) during the development of LVH. Therefore, manipulation of the expression of these molecules could offer alternative targets for treatment of LVH occurring as a result of myocardial stress or ischaemic heart disease.

6.1. Hypothesis for the involvement of cell cycle regulatory molecules in the development of LVH

In order to explain cardiac myocyte growth by hypertrophy in terms of cell cycle control, we have formulated the following hypothesis (see Fig. 2). We propose that the majority of normal adult cardiac myocytes are arrested in the $G_0/G_1$ phases of the cell cycle, as demonstrated by FACS analysis [76]. Consistent with this $G_1$ arrest, we have shown a decrease in the expression and activities of certain positive regulators of the cell cycle (cyclins and CDKs) in adult myocytes, compared with levels found in foetal or neonatal cells, concomitant with an upregulation in the expression of $p21$ and $p27$ KIP/CIP CDK1 molecules [76,93]. In order for hypertrophic growth to occur, the cell must proceed through $G_1$ to synthesise certain mRNAs and proteins that are necessary for DNA synthesis or binucleation [1–5,20]. In order for this to occur, the cyclin–CDK:CDKI ratios must alter, such that the activities of certain positive regulators override the inhibitory effects of the CDKIs (Fig. 2). Our recent studies have demonstrated that such an effect occurs transiently, but significantly, during the development of pressure overload-induced LVH, since mRNA and protein levels of $p21$ and $p27$ were down-regulated during the active LV growth phase (3–14 days after the imposition of pressure overload) [19]. Moreover, we have demonstrated recently a concomitant upregulation in the expression and activities of certain cyclin–CDK complexes during this period of LV growth that is consistent with cell cycle progression during the development of LVH [99]. Once cells have synthesised the necessary mRNAs and proteins for hypertrophic growth, some will enter the $S$ phase and undergo DNA synthesis and binucleation [1–5,20]. Since, by definition, the hypertrophied myocyte is unable to undergo mitosis, any cells that enter the $S$ phase must arrest in $G_2$, where we propose that the balance of positive:negative regulators returns to that seen in normal adult myocytes (Fig. 2). In support of this hypothesis, we have shown that the changes in protein expression and activities of cell cycle regulatory molecules following the imposition of pressure overload is transient and that they return to control levels when the adaptive LV growth is completed. Furthermore, FACS analyses have shown that the number of myocytes arresting in $G_2$ is enhanced significantly during the development of LVH, such that more than 25% of myocytes are found in the $G_2$ phase of the cycle 14 days after AC operation, whereas only ~15% of untreated or SH cells are found in $G_2$ at this same time point [99].

7. Summary

The precise mechanism(s) by which cell cycle-dependent molecules cause a progressive withdrawal of cardiac myocytes from the cell cycle remains to be determined. However, by carefully dissecting the possible molecules involved, we hope to understand fully the mechanisms involved in cardiac myocyte growth. Certainly, our initial findings suggest that certain CDKI molecules play a pivotal role by acting as a “brake”, which results in cardiac myocyte cell cycle arrest during development. Furthermore, we have demonstrated that the expression of certain CDKIs is tightly regulated during the development of LVH, thereby implicating these molecules in this process also. Manipulation of the expression of these molecules by antisense technology and/or overexpression of positive regulators in adult myocytes may enable us to overcome the block in cell cycle progression, such that we can reinitiate cell division in these cells in a controlled manner. Such an approach could be invaluable in encouraging healthy myocytes that surround an infarcted area to repopulate a damaged myocardial zone.

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