Notch-Dependent Cell Cycle Arrest Is Associated With Downregulation of Minichromosome Maintenance Proteins

Michela Noseda, Kyle Niessen, Graeme McLean, Linda Chang, Aly Karsan

Perturbation of the Notch signaling pathway has been implicated in the pathogenesis of human cardiovascular diseases, and animal models have confirmed the requirement of Notch during cardiovascular development. We recently demonstrated that Notch activation delays S-phase entry and contributes to endothelial contact inhibition. Minichromosome maintenance (MCM) proteins, components of the prereplicative complex (pre-RC), are essential for DNA replication. Here, we report that Notch-mediated cell cycle arrest is associated with downregulation of MCM2 and MCM6 in endothelial cells and human fibroblasts. Downregulation of MCM proteins is also observed on activation of C promoter binding factor (CBF1) and is mediated by inhibition of Rb phosphorylation, as demonstrated using a constitutively active Rb mutant. Although the effects of the Notch pathway are cell-type specific and context-dependent, in cell types where Notch has an antiproliferative effect, downregulation of MCM proteins may be a common mechanism to inhibit DNA replication.

Intercellular signaling mediated by Notch receptors is essential for proper development and homeostasis of the cardiovascular system; indeed, perturbations of the Notch pathway have been implicated in the pathogenesis of several cardiovascular diseases.1–3 We have recently shown that activation of Notch in endothelial cells has an antiproliferative effect which may explain the defects of vascular remodeling consequent to dysregulation of Notch activation.3,4 In endothelial cells, Notch delays progression toward S-phase through a mechanism that depends, at least in part, on improper subcellular localization of the cyclin D–cdk4 complex secondary to downregulation of p21Cip1.4 Given that this mechanism seems to be endothelial-specific and targeted downregulation of p21Cip1 by short interfering RNA induces a less efficient block of S-phase entry compared with Notch activation, we postulated that additional mechanisms may contribute to Notch-mediated cell cycle arrest.4

Minichromosome maintenance (MCM) proteins 2 to 7 form a complex with helicase activity and participate in the formation of prereplicative complexes (pre-RCs) that allow chromatin licensing to ensure that DNA replication initiates at specific sites.5 Thus, MCM proteins are essential for DNA replication and cell cycle progression.6,7 Indeed, inactivation of MCM2 in Drosophila reduces proliferation in the developing central nervous system and microinjection of antibodies targeting MCM3 and MCM2 inhibits DNA replication.5 Here, we identify repression of MCM2 and MCM6 as a mechanism of Notch-mediated cell cycle arrest.

Materials and Methods

Cell Culture

The human microvascular endothelial cell line, HMEC-1 (HMEC) and human umbilical vein endothelial cells (HUVEC) were cultured as previously described.4,4 Primary human foreskin fibroblasts (HFF) were cultured in Dulbecco’s modified Eagle’s medium with 10% heat inactivated fetal bovine serum (HyClone).

Plasmids and Gene Transfer

HUVEC and HFF were transduced using Amphi-Phoenix packaging cells.5 Expression of Notch4IC, Notch1IC, CBF1-VP16, and RbΔK11 proteins were confirmed by immunoblotting (data not shown). For a description of plasmids, see Material and Methods in the online data supplement at http://circres.ahajournals.org.

Immunoblotting and Immunofluorescence

Immunostaining was performed as previously described.4 For list of antibodies used see Material and Methods in the online data supplement.

Statistical Analysis

To determine statistical significance, a Student t test for comparison between 2 groups was used, whereas a 1-way ANOVA with a Tukey test was used for multiple comparisons. Statistical significance was taken at P<0.05.

Results and Discussion

To identify new pathways mediating the Notch antiproliferative effect, HMEC transduced with vector alone (HMEC-pLNCX) or vector encoding the active intracellular portion of the endothelial-specific Notch4 (HMEC-pLNC-Notch4IC) were analyzed using high-throughput immunoblotting (Power Blot, BD Pharmingen).7 MCM2 and MCM6 were consistently downregulated in the HMEC-pLNC-Notch4IC compared with HMEC-pLNCX (online Table I). As we have previously shown, fibronectin was found to be highly upregulated in HMEC-pLNC-Notch4IC (online Table I).8 These findings were validated in HUVEC transduced with empty vector, vector encoding activated Notch4 (Notch4IC) or Notch1 (Notch1IC), because both Notch family members block S-phase entry.4 Both active Notch4 and Notch1 downregulated MCM2 and MCM6 (Figure 1A and online Figure

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Together with previous studies, these data suggest that Notch-mediated endothelial cell cycle arrest is effected by multiple pathways that impinge on DNA replication via downregulation of members of the pre-RC.

Binding of NotchIC to CBF1, the best described effector of the Notch pathway, induces derepression/activation of CBF1 target gene transcription. Notch activation in endothelial cells triggers CBF1 activity, and the Notch pathway induces endothelial quiescence partly by a mechanism that impedes phosphorylation of Rb. We used a constitutively-active form of CBF1, obtained by fusing the CBF1 cDNA with the transcriptional activation domain of herpes virus protein, VP16 (CBF1-VP16), to determine whether Notch-mediated cell cycle arrest could be mimicked by CBF1 activation alone. HUVEC-CBF1-VP16 showed reduction of the proportion of cells entering S-phase and of cells expressing phosphorylated Rb, compared with the HUVEC-vector, suggesting that CBF1-VP16 impedes cell cycle progression at least in part via inhibition of Rb phosphorylation (Figure 1B). As well, CBF1-VP16 markedly repressed MCM2 and MCM6 expression (Figure 1C and online Figure S1B). These data demonstrate that activation of CBF1 is sufficient to inhibit Rb phosphorylation and repress MCM2 and MCM6.

MCM proteins are essential for progression of the cell cycle, but the expression of MCM2 and MCM6 in proliferating and arrested endothelial cells has not been examined. Rb phosphorylation can be used as an indicator of actively proliferating cells, because it is required for progression toward S-phase. Rb phosphorylation was abolished in contact-inhibited compared with logarithmically growing HUVEC. Rb hypophosphorylation directly correlated with the downregulation of MCM2 and MCM6 (Figure 2A). Thus, expression of MCM2 and MCM6 is regulated in concert with Rb phosphorylation and the proliferative status of endothelial cells.
Overexpression of E2F transcription factors, which are released on Rb phosphorylation, induce MCM expression.\textsuperscript{11,12} Because both Notch activation and activated CBF1 inhibit Rb phosphorylation, we tested whether persistently hypophosphorylated Rb, which does not release E2F, is sufficient to repress MCM2 and MCM6 expression. Expression of a phosphorylation-resistant Rb mutant with 11 serine/threonine to alanine substitutions (RbΔK11) inhibits cell proliferation and E2F transcriptional activity similar to the constitutively-active and hypophosphorylated native Rb (data not shown).\textsuperscript{13} Attenuation of phosphorylation of endogenous Rb in endothelial cells expressing RbΔK11, using an antibody specific for 2 phospho-acceptor sites that have been mutated in RbΔK11, confirmed that this construct maintains Rb in a hypophosphorylated state (Figure 2B). HUVEC transduced with RbΔK11 (HUVEC-RbΔK11) show downregulation of MCM2 and MCM6 compared with cells transduced with vector alone (HUVEC-vector) (Figure 2C). Hence, Notch-mediated CBF1-dependent inhibition of Rb phosphorylation appears sufficient to mediate downregulation of MCM2 and MCM6.

The effects of Notch activation on proliferation can be stimulatory or inhibitory depending on the cell type, and the mechanisms mediating cell cycle inhibition can be cell-type specific.\textsuperscript{4,14} Thus, we sought to determine whether the Notch/CFB1/Rb-dependent mechanism impeding cell cycle progression is conserved in fibroblasts. Active Notch1 reduced Rb phosphorylation, reduced the proportion of HFF entering S-phase, and downregulated MCM2 and MCM6 (online Figure SIC, SIIA, and SIIB). Similarly, constitutively active CBF1-VP16 inhibited S-phase entry and Rb phosphorylation, as well as downregulated MCM2 and MCM6 expression (online Figure SID, SIIC, and SIID). Finally, we confirmed that RbΔK11 also downregulates MCM2 and MCM6 in HFF (online Figure SID and SIIE). Together these results suggest that inhibition of Rb phosphorylation and downregulation of MCM proteins via a CBF1-dependent mechanism are conserved elements of Notch-mediated cell cycle arrest in at least 2 different cell types.

Taken together our results suggest that Notch activation triggers an axis of events that, through the activation of CBF1, interferes with Rb phosphorylation and results in downregulation of MCM2 and MCM6 in endothelial cells and fibroblasts. However, fibroblasts do not show suppression of p21\textsuperscript{CIP1} after Notch activation (data not shown). Hence, repression of MCM proteins may represent a common downstream mechanism for Notch-mediated cell cycle arrest in some cell types. It remains to be established what the effect on MCM expression is in cell types that are stimulated to grow after Notch activation. However, Notch- and CBF1-dependent hypophosphorylation of Rb with consequent downregulation of MCM proteins could be a downstream effect of the arrest in G0/G1 caused by mechanisms that remain to be elucidated. Nevertheless, these results show that there is a functional correlation between 2 highly conserved cellular pathways: the Notch pathway that regulates cell fate through an intercellular signaling mechanism and the ancestral MCM proteins that are essential for initiation of DNA replication.

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**References**


**Key Words:** minichromosome maintenance proteins | cell cycle | Notch | endothelial cells | retinoblastoma protein
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Supplementary material, Material and Methods

**Antibodies used for immunoblotting and immunofluorescence staining.** Anti-MCM2, anti-MCM6 and anti-Rb (clone G3-245) antibodies were all obtained from BD Pharmingen (Bedford, MA). Anti-α-tubulin antibody was purchased from Sigma. The anti-BrdU antibody conjugated with Alexa 594 was purchased from Molecular Probes. The anti-phospho Rb antibody (specific for phosphorylation on Ser807/811 of human Rb corresponding to Ser800/804 of murine Rb) was from Cell Signaling Technologies (Beverly, MA).

**Plasmids.** pLNCX and pLNC-Notch4IC plasmids were previously described (Leong KG et al; *Mol Cell Biol*. 2002;22:2830-41.) To transduce HUVEC and HFF we used constructs encoding the C-terminal HA-tagged Notch4IC, Notch1IC and Flag-tagged CBF1-VP16 subcloned into the MSCV-IRES-YFP vector (MIY). cDNA encoding RbΔK11 (gift of Dr. E. Zacksenhaus, University of Toronto, Toronto, ON) was also subcloned into MIY.
Supplementary data, Table 1

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold change&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
</tr>
<tr>
<td>MCM2</td>
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</tr>
<tr>
<td>MCM6</td>
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</tr>
<tr>
<td>Fibronectin</td>
<td>(+)/0&lt;sup&gt;3&lt;/sup&gt;</td>
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<sup>1</sup>Fold change is a semiquantitative value that represents the general trend in protein changes representing expression in HMEC-pLNC-Notch4IC relative to HMEC-pLNCX.

<sup>2</sup>0/(+) represents absence of a protein in HMEC-pLNC-Notch4IC versus presence in HMEC-pLNCX (fold change unmeasurable).

<sup>3</sup>(+)/0 represents presence of a protein in HMEC-pLNC-Notch4IC versus absence in control cells (fold change unmeasurable).
Densitometric analysis shows relative expression of MCM2 and MCM6. (A and B) HUVEC transduced with vector alone, Notch4IC, Notch1IC or CBF1-VP16 were lysed and tested by immunoblotting for expression of MCM2, MCM6 and tubulin as a loading control. Expression was analyzed by densitometry and quantification of MCM2 and MCM6 was normalized to tubulin. (B and C) HFF transduced with vector, Notch1IC, CBF1-VP16 and RbΔK11 were analyzed as described for HUVEC. Graphs represent the mean ± SEM of at least three experiments.*P<0.05 compared to vector.
Notch activation inhibits proliferation of human fibroblasts via CBF1- and Rb-dependent repression of MCM2 and MCM6.

(A) HFF-vector and HFF-Notch1IC were examined by immunofluorescence for BrdU incorporation and Rb phosphorylation. The proportion of cells incorporating BrdU and expressing phosphorylated Rb (ppRb) is presented relative to HFF-vector (mean ± SD). (B) Immunoblotting for MCM2, MCM6 and α-tubulin. (C) Immunofluorescence for BrdU incorporation and expression of phosphorylated Rb (ppRb) in HFF transduced with empty vector or CBF1-VP16. (D) Lysates from HFF-vector or HFF-CBF1-VP16 were analyzed by immunoblotting for MCM2, MCM6 and α-tubulin. (E) Lysates from HFF-vector or HFF-RbΔK11 were analyzed by immunoblotting for MCM2, MCM6 and α-tubulin.