Absence of Caprin-1 Results in Defects in Cellular Proliferation

Bin Wang, Muriel D. David and John W. Schrader

*J Immunol* 2005; 175:4274-4282; doi: 10.4049/jimmunol.175.7.4274

http://www.jimmunol.org/content/175/7/4274

---

**References**

This article cites 29 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/175/7/4274.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Absence of Caprin-1 Results in Defects in Cellular Proliferation

Bin Wang, Muriel D. David, and John W. Schrader

Cytoplasmic activation/proliferation-associated protein-1 (Caprin-1) is a cytoplasmic phosphoprotein that is the prototype of a novel family of highly conserved proteins. Its levels, except in the brain, are tightly correlated with cellular proliferation. We disrupted caprin-1 alleles in the chicken B lymphocyte line DT40 using homologous recombination. We readily obtained clones with one disrupted allele (31% of transfectants), but upon transfection of heterozygous cells we obtained a 10-fold lower frequency of clones with disruption of the remaining allele. Clones of caprin-1-null DT40 cells exhibited marked reductions in their proliferation rate. To obviate the problem that we had selected for the lack of Caprin-1, we generated clones of DT40 cells heterozygous for the caprin-1 gene in which, during disruption of the remaining wild-type allele of the chicken caprin-1 gene, the absence of endogenous Caprin-1 would be complemented by conditional expression of human Caprin-1. Suppression of expression of human Caprin-1 resulted in slowing of the proliferation rate, due to proliferation of the G1 phase of the cell cycle, formally demonstrating that Caprin-1 was essential for normal cellular proliferation. The Journal of Immunology, 2005, 175: 4274–4282.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Materials and Methods

Cell culture

DT40 cells were maintained in log-phase proliferation at densities between 104 and 106 cells/ml, in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 1% chicken serum, and 50 μM 2-ME.

Cloning of chicken Caprin-1 cDNA

A 2.1-kb cDNA-encoding chicken Caprin-1 was amplified by RT-PCR using RNA from DT40 cells and the primers 5'-GGGATCCCATCATGC CCTCGGCTACCCGACC-3' (sense) and 5'-GGTTAATTCCTT GCTAGCCTTCA-3' (antisense).

Targeting constructs

The 8.2-kb genomic region of the caprin-1 locus shown in Fig. 2A was amplified by PCR, using DT40 genomic DNA as a template and the primers S4, 5'-CTTGAAGATCCAGCAGAAGAAGA-3' and A5S, 5'-TCCTTCTACTGCTGAAGT-3', and then subcloned into pPNT-NHS14 M 2-ME.

Received for publication March 1, 2005. Accepted for publication July 20, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Canadian Institutes of Health Research and fellowships to M.D.D. and B.W. from the Canadian Arthritis Network. M.D.D. is currently supported by a grant from the Arthritis Society of Canada.

2 Address correspondence and reprint requests to Dr. John W. Schrader, Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada.

3 Address correspondence and reprint requests to Dr. John W. Schrader, Biomedical Research Centre, University of British Columbia, 2222 Health Sciences Mall, Vancouver, British Columbia, V6T 1Z3, Canada. E-mail address: john@brc.ubc.ca

4 Abbreviations used in this paper: Caprin, cytoplasmic activation/proliferation-associated protein; Dox, doxycycline; G3BP-1, Ras-GTPase-activating protein Src homology 3 domain-binding protein; PI, propidium iodide; PKB, protein kinase B.

Copyright © 2005 by The American Association of Immunologists, Inc.
respective... Finally, the phosphoglycerate kinase promoter-neomycin cassette of these pPN'T-primer derivatives was replaced with cassette, oriented in the direction opposite to the arms, containing a chicken β-actin promoter driving expression of the purocycin resistance gene, for the pPN'T2-puro (S7-AS7) construct, or of the blasticidin resistance gene, for the pPN'T2-bsr (S8-AS8) and the pPN'T3-bsr (S53-AS1) constructs.

**DNA transformation and gene disruption**

A total of 10^7 DT40 cells was electroporated with 50 μg of NdeI-linearized pPN'T2-puro construct at 55 V, 50 μA, using a Gene Pulser (Bio-Rad), and selected in presence of 1 μg/ml purocycin (Sigma-Aldrich) for 10–14 days. Drug-resistant colonies were further selected in 2 μM ganciclovir (Sigma-Aldrich) for 3 days. The heterozygous caprin-1/-/- clones were identified by PCR amplification of the caprin-1-purocycin fusion gene, using the primers S4 and Puro (5'-CAGCGCCCGACCGAAAGGAGCG/H11032/ H11001/H11002/H11003) and AS1 (5'-CAGCTGC-3'). To generate caprin-1-null DT40 cells, a heterozygous caprin-1/-/- clone was transfected with the pPN'T2-bsr (first attempt) or the pPN'T3-bsr constructs. Clones were selected in presence of 15 μg/ml blasticidin-S (In vitrogen Life Technologies) for 10–14 days, and then in presence of 2 μM ganciclovir for 3 days. Genomic DNA was analyzed by PCR using the primers S4 and AS10 (5'-TGTCACTTCCTGGTATTTTGA/H11032/ H11001/H11002) and AS6 (5'-CGT). To isolate chicken Caprin-1 cDNA, we searched the Bursal Developmental Database (http://www.bdb.org) and identified several expressed sequence tag sequences with high homology to parts of the mouse and human Caprin-1 cDNAs described previously (1). RNA from DT40 cells was used as a template for PCR. The amplified fragments were sequenced (GenBank accession AJ475194). It exhibited 81% identity with human and mouse Caprin-1 cDNAs, respectively, and the predicted protein of 709 aa that it encoded exhibited 92% identity with both human (709 aa) and mouse (707 aa) Caprin-1 (Fig. 1). These data indicated that this cDNA encoded the chicken ortholog of the human Caprin-1 described previously (1), and revealed a high level of conservation of Caprin-1 between species.

**Generation of caprin-1-deficient DT40 cells**

We exploited the unusually high rate of homologous recombination in the DT40 line of chicken B lymphocytes to generate caprin-1-deficient cells and investigate its cellular functions. To clone parts of the chicken caprin-1 gene, we used genomic DNA from DT40 cells as a template for PCR. The amplified fragments were cloned in a series of targeting constructs based on pPN'T. In the pPN'T2-puro construct, an actin-promoter-purocycin cassette was flanked by fragments corresponding to introns 3 and 7 of the caprin-1 gene. The pPN'T2-bsr construct was identical, except that the purocycin resistance gene was replaced by a blasticidin resistance gene. Homologous recombination of the caprin-1 gene with the pPN'T2-puro or the pPN'T2-bsr constructs would result in deletion of the region encompassing exons 4–7 of the chicken caprin-1 gene (encoding 80% of the HR-1 region of Caprin-1, TCAACACCTCCTTCAGG-3') were used as forward and reverse primers, respectively. For amplification of chicken Caprin-1 cDNA, we used the forward primer 5'-CCAGCGGGAGAGGAGGACTCATTTG-3' and the reverse primer 5'-GGTGCAGGATGGTGGATAAC-3'. For amplification of human Caprin-1 cDNA, we used the forward primer 5'-ACGGTGCATGATGCTATATTG-3' and the reverse primer 5'-CCCCGTATGAGCGCAGATTTACC-3'. After a 3-min denaturation step at 95°C, amplification reactions of Caprin-1 cDNAs were conducted during 45 (chicken) or 40 (human) cycles of successive incubations at 95°C for 5 s, 60°C (chicken) or 65°C (human) for 5 s, and 72°C for 18 s. Chicken hoxypoxan guanine phosphoribosyltransferase cDNA was amplified as an internal control using the forward primer 5'-TCATGGAGAGGGGAAGAGCGAG-3' and the reverse primer 5'-GGGCAGCAATGTGGCGTAGAGTC-3'. Statistical analysis of the quantification of relative levels of mRNA expression by real-time PCR was conducted using the software REST-XL version 2 and the Pair-Wise Fixed Reallocation Randomization Test (12).

**Cell cycle analysis**

Cells were synchronized at metaphase by treatment with 0.5 μg/ml nocodazole for 10 h, and then washed thoroughly. At the indicated times, cells were resuspended at 10^5 cells/ml in PBS containing 50 μg/ml pro-pidium iodide (PI) and 0.1% sodium azide, mixed with an equal volume of Vindelov’s PI staining solution (0.01 M Tris base, 0.01 M NaCl, 700 U of RNase, 7.5 × 10^3 M PI, 0.1% Nonidet P-40), and incubated for 30 min on ice. Flow cytometry was performed using a BD Biosciences FACSCalibur. Asynchronous DT40 cells were pulse labeled with 10 μM BrdU, as previously described (13). A BrdU Flow kit (BD Pharmingen) was used to determine the cell cycle distribution of BrdU-labeled cells by flow cytometry.

**Cell counting**

Cell suspensions were mixed with a predetermined number of 10-μm microspheres (Polysciences). The ratio of viable cells to microspheres was assessed by flow cytometry.

**Results**

Isolation of chicken full-length Caprin-1 cDNA

To isolate chicken Caprin-1 cDNA, we searched the Bursal Expressed Sequence Tag Database (http://swallow.gsdf.doe/dt40, html) and found several expressed sequence tag sequences with high homology to parts of the mouse and human Caprin-1 cDNAs described previously (1). RNA from DT40 cells was used as a template for RT-PCR using primers designed in these conserved sequences. The PCR product was cloned and sequenced (GenBank accession AJ475194). It exhibited 81% identity with human and mouse Caprin-1 cDNAs, respectively, and the predicted protein of 702 aa that it encoded exhibited 92% identity with both human (709 aa) and mouse (707 aa) Caprin-1 (Fig. 1). These data indicated that this cDNA encoded the chicken ortholog of the human and mouse Caprin-1 described previously (1), and revealed a high level of conservation of Caprin-1 between species.
from aa 87 to 268), and would render the cells resistant to puromycin or blasticidin, respectively (Fig. 2A). Nonhomologous recombination events (i.e., random integration of these constructs) would result in sensitivity of transfected cells to ganciclovir, due to expression of the thymidine kinase gene present in all of the pPNT-derived targeting constructs.

Wild-type DT40 cells were transfected with the pPNT2-puro construct, and selected with both puromycin and ganciclovir. Double drug-resistant clones were examined by PCR using the forward primer S4, specific for exon 3 of the caprin-1 gene, and the reverse primer Puro, specific for the puromycin resistance gene. Successful disruptions of a caprin-1 allele were detected by the presence of a 2.2-kb band, corresponding to a chimeric fragment containing elements of both the caprin-1 (exon 3 and intron 3) and the puromycin resistance genes (Fig. 2B). Several such clones were obtained.

**FIGURE 1.** Conservation of the sequences of chicken, human, and mouse Caprin-1 proteins. Shown are alignments of the predicted amino acid sequences of chicken Caprin-1 (GenBank accession AY745194), human Caprin-1 (GenBank accession DAA01121.1), and mouse Caprin-1 (GenBank accession DAA01122.1), made using the Clustal method with the Macvector software (Accelrys). Identical residues are darkly shaded, and similar residues lightly shaded.
To generate homozygous (caprin-1−/−) cells, one of the clones that were heterozygous for caprin-1 (caprin-1+/−) was transfected with the pPNT2-bsr targeting construct. Subclones were selected for resistance to blasticidin and ganciclovir, and disruption of the remaining caprin-1 allele was identified by PCR using the primers S4 and AS10, specific for the exons 3 and 4 of the caprin-1 gene, respectively. Thus, PCR of genomic DNA from caprin-1+/−, but not caprin-1−/− DT40 cells would yield a fragment of 1.9 kb, corresponding to the region encompassing exons 3–4 of the wild-type caprin-1 gene.

However, we analyzed 130 clones without finding a single clone in which the remaining caprin-1 allele had been disrupted. This result suggested that either the pPNT2-bsr plasmid yielded an unexpectedly lower efficiency of targeting than the pPNT2-puro construct, or, more likely, that cells in which both caprin-1 alleles had been disrupted had an impaired ability to proliferate and/or survive.

To obtain a higher frequency of homologous recombination (4), we lengthened one of the regions in the pPNT2-bsr targeting construct that were homologous to the caprin-1 gene. Thus, in the new targeting construct, pPNT3-bsr, we replaced the 3’ arm of the pPNT2-bsr plasmid that corresponded to the 1.3-kb intron 7 of the caprin-1 gene, with a 3.3-kb fragment encompassing intron 6, exon 7, and intron 7. We transfected this construct into caprin-1+/− cells, selected the stable transfectants in presence of blasticidin and ganciclovir, and screened for disruption of the remaining wild-type caprin-1 allele by PCR using the primers S4 and AS10. Using this strategy, we obtained a total of four clones in the case of which PCR amplification of genomic DNA using these primers failed to yield the 1.9-kb fragment corresponding to the region encompassing exons 3–4 of the wild-type caprin-1 gene (Fig. 2C). PCR with the primers S7 and Puro was used as an internal positive control, to verify the quality of genomic DNA derived from each clone (data not shown). We also used RT-PCR with the primers S53 and AS6, specific for exons 5 and 7, respectively, to confirm that these clones did not express Caprin-1 mRNA (Fig. 2D). These data indicated that, in these clones, we had successfully disrupted both alleles of the caprin-1 gene.

Low frequency of recovery of caprin-1−/− clones

As summarized in Table I, homologous recombination in wild-type DT40 cells of one or the other of the caprin-1 alleles with the pPNT2-puro construct occurred in 2 of the 28 recovered clones that we analyzed (7% of the total number of clones). However, in similar experiments using caprin-1+/− cells and the very similar pPNT2-bsr construct, we analyzed 130 clones without finding a single clone in which the remaining caprin-1 allele had been disrupted. With the pPNT3-bsr construct, we obtained a much higher frequency of homologous recombination events than with the pPNT2-bsr construct. Thus, after transfection of pPNT3-bsr into wild-type DT40 cells and selection in presence of blasticidin and ganciclovir, and disrupted of the remaining caprin-1 allele, we obtained a total of four clones in the case of which PCR amplification of genomic DNA using these primers failed to yield the 1.9-kb fragment corresponding to the region encompassing exons 3–4 of the wild-type caprin-1 gene (Fig. 2C). PCR with the primers S7 and Puro was used as an internal positive control, to verify the quality of genomic DNA derived from each clone (data not shown). We also used RT-PCR with the primers S53 and AS6, specific for exons 5 and 7, respectively, to confirm that these clones did not express Caprin-1 mRNA (Fig. 2D). These data indicated that, in these clones, we had successfully disrupted both alleles of the caprin-1 gene.

![FIGURE 2. Generation of caprin-1-deficient DT40 clones. A. Schematic representation of the partial chicken caprin-1 locus, the three targeting constructs (pPNT2-puro, pPNT2-bsr, and pPNT3-bsr), and the structure of the targeted loci. The open boxes and the numbers indicate exons of Caprin-1. Arrowheads indicate localization of primer sites for PCR. Genomic DNA from wild-type (+/+) heterozygous (+/−), and homozygous mutant (−/−) clones was isolated and examined by PCR using the primers S4 and Puro (B), and S4 and AS10 (C). D, RT-PCR analysis of mRNA from caprin-1+/+, caprin-1+/−, and caprin-1−/− clones. Total RNA was isolated from the cells and amplified by RT-PCR using the primers S53 and AS6.](http://www.jimmunol.org/)

Table I. Reduced frequency of recovering caprin-1-null clones

<table>
<thead>
<tr>
<th>Construct</th>
<th>Target Cell</th>
<th>Integrations at</th>
<th>Clones Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPNT2-puro</td>
<td>+/+</td>
<td>2 (7.3%) 0 26</td>
<td>28</td>
</tr>
<tr>
<td>pPNT2-bsr</td>
<td>+/-</td>
<td>ND 0 130</td>
<td>130</td>
</tr>
<tr>
<td>pPNT3-bsr</td>
<td>+/-</td>
<td>10 (31.3%) 0 22</td>
<td>32</td>
</tr>
<tr>
<td>pPNT3-bsr</td>
<td>+/-</td>
<td>15 (16.3%) 3 (3.2%) 74</td>
<td>92</td>
</tr>
</tbody>
</table>

*Random integrations.

| Replaces pPNT2-puro cassette. |
ganciclovir, we observed that 10 of the 32 clones screened (31%) exhibited a disrupted caprin-1 allele. However, when we transfected this construct into caprin-1<sup>−/−</sup> cells, we once again observed a strikingly lower recovery of clones with disruption of the remaining wild-type caprin-1 allele. Thus, only 3 of the 92 (3.2%) blasticidin- and ganciclovir-resistant clones analyzed by PCR were caprin-1<sup>−/−</sup>. In contrast, 15 of these 92 clones (16%) exhibited homologous recombination at the allele that had already been targeted (i.e., the second recombination event had resulted in replacement of the puromycin resistance cassette of the original targeting construct by the blasticidin resistance gene of the incoming targeting construct). Of note, the caprin-1 allele that had already been disrupted by the first targeting construct (and was therefore devoid of the region encompassing intron 6 to exon 7) exhibited a much shorter region of homology with the 3′ arm of the second targeting construct (intron 6–7 of the caprin-1 gene) than did the remaining wild-type caprin-1 allele. Thus, this result demonstrated that the homologous recombination process per se was still efficient in the caprin-1<sup>−/−</sup> heterozygous cells, and strongly suggested that the extremely low frequency at which caprin-1<sup>−/−</sup> clones were obtained resulted from a greatly reduced ability of caprin-1<sup>−/−</sup> cells to proliferate into identifiable clones.

Defective proliferation of caprin-1<sup>−/−</sup> cells

We examined the effect of absence of Caprin-1 on the ability of DT40 cells to form colonies in 0.3% agar. As shown in Fig. 3A, caprin-1<sup>−/−</sup> DT40 cells (clones 1 and 2) showed a marked decrease in their efficiency at forming colonies as compared with wild-type or caprin-1<sup>+/−</sup> cells. Furthermore, most of the caprin-1<sup>−/−</sup> colonies were much smaller than those formed by wild-type or heterozygous cells (Fig. 3A).

Moreover, as shown in Fig. 3B, while the proliferation rates of caprin-1<sup>+/+</sup> and wild-type DT40 cells were indistinguishable, cells from all four of the independent caprin-1<sup>−/−</sup> clones obtained increased in number much more slowly than their wild-type or caprin-1<sup>+/−</sup> counterparts. In only one of these four caprin-1<sup>−/−</sup> clones (clone 2 in Fig. 3B), this decrease in cell numbers was contributed to by an increase in cell death. Thus, in three of the four caprin-1<sup>−/−</sup> clones (including clone 1 shown in Fig. 3B), we observed no significant number of apoptotic or necrotic cells, as assessed by trypan blue staining and by microscopy, or by PI staining and flow cytometry (data not shown). The variation observed between these caprin-1<sup>−/−</sup> clones highlighted the possibility that the clones that we had successfully recovered might have been selected for genetic changes that partially compensated for the absence of Caprin-1. We investigated the possibility that the absence of Caprin-1 may have led to compensatory changes in the expression of Caprin-2, which may display some functional redundancy with Caprin-1. Using RT-PCR and real-time PCR, we showed that DT40 cells expressed chicken Caprin-2. However, there were no significant changes in the levels of Caprin-2 mRNA in the caprin-1<sup>−/−</sup> clones of DT40 (data not shown).

Generation of caprin-1<sup>−/−</sup> cells conditionally expressing human Caprin-1

To rule out the possibility that the relatively few caprin-1-null clones we obtained had been selected for mutations that partially compensated for the lack of Caprin-1, we generated clones in which targeting of the second endogenous caprin-1 allele could be undertaken in the presence of exogenous human Caprin-1. In that chicken and human Caprin-1 are highly conserved (Fig. 1), we hypothesized that exogenous expression of human Caprin-1 would functionally complement absence of endogenous Caprin-1 in chicken cells.

FIGURE 3. Cloning efficiencies and proliferation rates of caprin-1<sup>+/+</sup>, caprin-1<sup>−/−</sup>, and caprin-1<sup>+/−</sup> DT40 clones. A, caprin-1<sup>+/+</sup>, caprin-1<sup>−/−</sup>, and caprin-1<sup>+/−</sup> cells were plated at 100 cells/ml in complete culture medium containing 0.3% agar and cultured for 8 days. Numbers of clones of four or more cells were counted under a microscope and classified as large (>20 cells) or small (<20 cells). Numbers of large and small colonies are shown in white and black on the histogram, respectively. B, caprin-1<sup>+/+</sup>, caprin-1<sup>−/−</sup>, and caprin-1<sup>+/−</sup> cells were seeded at 100 cells/ml in complete culture medium, using 24-well tissue culture plates. Viable cells were counted 4 days later by flow cytometry. Shown are the results of three independent experiments performed in triplicates. Error bars correspond to the SEM.

We transfected one of the caprin-1<sup>+/+</sup> clones described above with a plasmid coding for a trans activator whose activity could be inhibited by treatment of cells with Dox, and then with a plasmid allowing expression of human Caprin-1 under the control of a promoter regulated by this trans activator (see flow chart in Fig. 4). The remaining wild-type allele of the chicken caprin-1 gene was
then disrupted by homologous recombination using the pPNT2-bsr targeting construct, in absence of Dox (i.e., allowing expression of human Caprin-1). The clones obtained after selection were designated rescued caprin-1⁻/⁻ (R-caprin-1⁻/⁻). When R-caprin-1⁻/⁻ cells were cultured for 4 days in medium containing 1 μg/ml Dox, expression of the exogenous human Caprin-1 was reduced to levels that were undetectable by immunoblotting (Fig. 5A). We also confirmed by real-time PCR that treatment with Dox for 96 h resulted in a 295-fold decrease in levels of human Caprin-1 mRNA (data not shown).

Analysis of cloning efficiency and proliferation rate of R-caprin-1⁻/⁻ cells

By transfecting clones of caprin-1⁺/⁺ cells that expressed exogenous human Caprin-1 using the pPNT2-bsr targeting construct, we readily obtained clones in which the remaining chicken caprin-1 allele had been disrupted. This contrasted with our difficulty in obtaining caprin-1⁻/⁻ DT40 cells in the absence of human Caprin-1 (Table I). This result strongly suggested that, as anticipated, human Caprin-1 had substituted for the endogenous chicken Caprin-1, and that the low efficiency of obtaining caprin-1⁻/⁻ clones from DT40 cells that lacked compensatory human Caprin-1 reflected the deleterious effect of absence of Caprin-1 on cloning efficiency. To test this hypothesis, we assessed the effects of suppressing expression of human Caprin-1 on the cloning efficiency of R-caprin-1⁻/⁻ cells. As shown in Fig. 5B, pretreatment of R-caprin-1⁻/⁻ cells for 96 h with Dox reduced their subsequent cloning efficiency by 50%, but had no significant effect on the cloning efficiency of parental DT40 cells. Thus, the lowered cloning efficiency of cells lacking Caprin-1 was likely to have accounted for much of the difficulty that we had in obtaining caprin-1⁻/⁻ clones by our first strategy.

To investigate the effects of absence of Caprin-1 on cellular proliferation, R-caprin-1⁻/⁻ cells and, as controls, wild-type and R-caprin-1⁺/⁺ DT40 cells were cultured for 12 days with or without Dox. Every second day, cells were counted and resuspended in fresh medium (with or without Dox) at a constant density (2 × 10⁶ cells/ml), to maintain exponential cell proliferation. In absence of Dox, wild-type, R-caprin-1⁺/⁺, and R-caprin-1⁻/⁻ cells multiplied at a similar rate, confirming that the expression of human Caprin-1 effectively complemented the absence of chicken Caprin-1 (Fig. 5C). During the first 3–4 days, the presence of Dox had only a marginal effect on the proliferation of any of these cells, consistent with our observation that suppression of levels of human Caprin-1 required 3–4 days of treatment with Dox. However, between days 4 and 12, when expression of human Caprin-1 was undetectable (Fig. 5A), the presence of Dox resulted in an 86% decrease in the cumulative numbers of R-caprin-1⁻/⁻ cells generated. Dox had no significant effect on the proliferation rate of parental DT40 cells throughout the experiment. These data indicated that the absence of Caprin-1 led to an increase in the cell-doubling time of ~20% (Fig. 5D). The observation that Dox-induced repression of expression of human Caprin-1 in chicken cells lacking endogenous Caprin-1 impaired their proliferation confirmed that the proliferative defect that we had observed in the caprin-1⁻/⁻ clones was indeed due to lack of Caprin-1. Suppression of expression of human Caprin-1, following treatment of R-caprin-1⁻/⁻ cells with Dox, did not increase the frequency of apoptotic cells (data not shown). Taken together, these results demonstrate that, at least in DT40 cells, expression of Caprin-1 is essential for normal cellular proliferation, but not for cellular viability.

Absence of Caprin-1 lengthens the cell cycle by prolonging the G₁ phase

To investigate how absence of Caprin-1 affected cell cycle progression, wild-type or R-caprin-1⁻/⁻ cells that had been pretreated with Dox for 4 days or left untreated were synchronized at the metaphase with nocodazole. They were then released from this block by thorough washing and resuspended in complete culture medium, with or without Dox. At successive time points, the percentage of cells in each phase of the cell cycle was determined by flow cytometry. As shown in Fig. 6A, while treatment with Dox failed to affect the progression of wild-type DT40 cells through the cell cycle, it resulted in a significant delay in the G₁ to S phase transition of R-caprin-1⁻/⁻ cells, with some of these cells remaining in G₁ at the end of the experiment. We also determined the percentages of cells in the different phases of the cell cycle in unsynchronized wild-type or R-caprin-1⁻/⁻ cells that had been pretreated with Dox for 4 days or left untreated. Although treatment with Dox did not affect the percentage of wild-type cells in the different phases of the cell cycle, it resulted in a significant increase in the percentage of R-caprin-1⁻/⁻ cells in the G₁ phase (data not shown), consistent with the results obtained with the synchronized cells.

To determine whether the lack of Caprin-1 also affected the progress of DT40 cells through other phases of cell cycle, we pulse labeled asynchronous DT40 cells with BrdU and followed the progress of these labeled cells through the cell cycle by flow cytometry. Comparison of the percentages of the labeled Dox-treated R-caprin-1⁻/⁻ cells in various stages of cell cycle at indicated time points with those of Dox-treated wild-type cells or of non-treated cells of either genotype showed that the lack of Caprin-1 did not slow progression from S to G2/M or from G2/M to the next G₁ phase (Fig. 6B). However, in agreement with our previous experiment using synchronization with nocodazole, the Dox-treated R-caprin-1⁻/⁻ cells exhibited a delay in progress from G₁ to early S phase (Fig. 6B).

Absence of Caprin-1 did not affect the phosphorylation of ERK or PKB triggered by ligation of the BCR for Ag

Activation of the BCR triggers many of the same signaling pathways important in the G₁ phase of the cell cycle. To explore the
role of Caprin-1 in BCR-mediated signaling, we stimulated wild-type and R-caprin-1−/− cells cultured in presence or absence of Dox with an anti-chicken IgM mAb (M4) and used immunoblotting to assess the phosphorylation of PKB (indicative of activation of the PI3K pathway) and ERK (indicative of activation of the MAPK pathways). There were no significant differences in the levels of phosphorylation of ERK or PKB triggered by ligation of the BCR between wild-type and R-caprin-1−/− cells treated with Dox (Fig. 7). These data indicate that the absence of Caprin-1 did not affect two of the major signaling pathways activated by stimulation of the BCR.

**Discussion**

These studies establish that the absence of Caprin-1 led to a significant defect in cellular proliferation that results from a specific prolongation of the G1 phase of the cell cycle (Fig. 6). All four of the caprin-1−/− clones that we isolated exhibited a slow rate of proliferation and a decreased cloning efficiency, characteristics that accounted for the unexpectedly low frequency of recovery of caprin-1−/− clones when the remaining wild-type allele was targeted in caprin-1−/− cells (Table I). In contrast, DT40 cells in which the remaining wild-type caprin-1 had been targeted in the presence of human Caprin-1 were normal in terms of their proliferation rate, cloning efficiency, and cell cycle progression. However, suppression of human Caprin-1 expression by treatment of these cells with Dox resulted in prolongation of the G1 phase of the cell cycle, slowing of proliferation, and a decrease in cloning efficiency. These experiments on the effects of conditional suppression of human Caprin-1 expression formally demonstrated that the phenotype we had observed in caprin-1−/− clones was indeed due to the absence of Caprin-1. Moreover, they showed that the function of Caprin-1 in promoting cell cycle progression and proliferation was phylogenetically conserved from chickens to humans, paralleling the strong conservation of primary structure of the respective proteins that we demonstrated (Fig. 1).

Our observations also show that Caprin-2 mRNA was expressed in DT40 cells, and that its levels did not change in the absence of Caprin-1. This suggests that physiological levels of Caprin-2 could not compensate for the absence of Caprin-1, and that these two proteins have discrete nonredundant functions.

The demonstration that Caprin-1 is needed for normal progression through G1/S is consistent with previous observations that levels of Caprin-1 protein increased when resting cells began to cycle, and decreased when cycling cells ceased to divide and differentiated (1). There was no evidence that the longer doubling times of cells lacking Caprin-1 were due to an increase in cell death. Thus, with the exception of one of the caprin-1−/− clones, we did not see any abnormally high accumulation of apoptotic or dead cells in cultures of caprin-1−/− or Dox-treated R-caprin-1−/− cells. Nor was there any evidence that cells lacking Caprin-1 exhibited increased sensitivity to apoptosis in response to a number of proapoptotic stimuli, including UV or x-ray irradiation, hyperosmolarity, and treatment with staurosporin (data not shown).
The molecular mechanisms underlying the defect in G1-S progression in caprin-1−/− cells are unknown. Caprin-1 is a phosphoprotein (1) that is phosphorylated by a kinase or kinases activated in cycling cells (14). However, inhibition of Caprin-1 expression in DT40 cells did not confer any changes in the rapid activation of the PI3K or ERK pathways following ligation of the BCR (Fig. 7), suggesting that Caprin-1 does not act upstream of these canonical signaling paths associated with cellular activation and proliferation. Some clues to the mechanisms through which Caprin-1 functions in the G1 phase of the cell cycle may come from the proteins with which it associates. Caprin-1 coprecipitated with unidentified phosphoproteins, two of ~66 kDa and two of ~33 kDa (1). We have tested the possibility that the binding partners of Caprin-1 might include known participants in G1-S progression. We observed that proliferating cell nuclear Ag and cyclin-dependent kinase 4, two proteins known to play crucial roles in cell cycle progression, coprecipitated with Caprin-1, although the stoichiometry was low, raising the possibility that the interaction was indirect or weak (Y. Xu, M. David, and J. Schrader, unpublished observations). We also used affinity-directed mass spectrometry to identify proteins coprecipitating with Caprin-1. One of them was Ras-GTPase-activating protein Src homology 3 domain-binding protein (G3BP-1), an RNA-binding protein (15–17) (M. David, P. Schubert, V. Lam, J. Kast, and J. Schrader, unpublished observations). Coprecipitation experiments confirmed that G3BP-1 interacted strongly with Caprin-1, making it a candidate for one of the 66-kDa phosphoproteins that we reported previously (1). Moreover, Caprin-1 and G3BP-1 were recently shown to copurify in a tight complex (18). Several lines of evidence link G3BP-1 with cell cycle progression. G3BP-1 only binds to p120RasGAP and localizes to the plasma membrane when p21Ras is activated (19). Expression of G3BP-1 induces entry to S phase in serum-deprived cells (20), and stimulation of cells with the growth factor Heregulin resulted in increased phosphorylation of G3BP-1 (21). Finally, G3BP-1 levels are increased in cancer, consistent with its increased expression in dividing cells and a role in cell division (20, 22). The precise role of G3BP-1 is unclear, although it binds to a series of mRNAs that encode proteins regulating cell proliferation, namely c-Myc (23) and CDK7 and CDK9 (24). Interestingly, Caprin-1 itself exhibits RGG motifs (M. David, unpublished observation), characteristic of RNA-binding proteins, in its C-terminal HR-2 domain. Although the arthropod Caprin-like proteins that are characterized by well-conserved HR-1 domains lack obvious
HR-2 domains (1), they nevertheless exhibit RGG motifs in their C-terminal regions, suggesting that interaction with RNA and RNA-binding proteins is an ancient function of HR-1 domain-containing proteins. In keeping with this notion, the HR-1 domain of Caprin-1 has recently been shown to bind RNA (26). It seems likely that Caprin-1, probably complexed with G3BP-1, is a component of RNA-protein complexes that regulate RNA metabolism and translation in dividing cells and in neurons.

The notion that Caprin-1 is involved in the regulation of RNA metabolism and translation would be consistent with our observation that the decreased proliferation of Caprin-1-deficient cells resulted from a prolongation of the phase of the cell cycle in which cells increase in size (G1 phase). There is increasing evidence that multiple signaling pathways that control proliferation, including the Ras, c-Myc, PI3K, and Rb pathways, act in part by up-regulating the synthesis of RNA and proteins (27). Future elucidation of the structural features of Caprin-1 required for binding to its various partners will enable the design of mutants of Caprin-1 for assessing the importance of these interactions in promoting cell cycle progression. The R-caprin-1+/− cells should prove valuable for investigating the ability of such mutants to complement the defect resulting from absence of expression of endogenous Caprin-1.

Gene-deletion strategies have revealed that the absence of proteins thought to have critical roles in the cell cycle, such as the cyclins (28) or the cyclin-dependent kinases (29), can have surprisingly mild or undetectable consequences for cell proliferation. Our finding that the absence of Caprin-1 led to prolongation of the G1 phase of the cell cycle indicates that, at least in B lymphocytes, Caprin-1 has an essential role in cell proliferation. The fact that, just as in B lymphocytes, the proliferation of thymocytes, T lymphoblasts, and hematopoietic progenitors correlates with increased levels of Caprin-1 suggests that Caprin-1 may also be critical for their proliferation, and thus the development and function of the entire immune system.

Acknowledgments
We thank Dr. Jean-Marie Buerstedde (Heinrich-Pette-Institute, Hamburg, Germany) for generously providing drug selection cassettes, and Dr. Peter A. Greer (Queen’s University Cancer Research Institute, Ontario, Canada) for providing the gene-targeting vector pPNT-NHS14. We also thank Andrew Johnson for his excellent technical assistance with flow cytometry.

Disclosures
The authors have no financial conflict of interest.

References