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Activation/Division of Lymphocytes Results in Increased Levels of Cytoplasmic Activation/Proliferation-Associated Protein-1: Prototype of a New Family of Proteins

Brock Grill,* Gary M. Wilson,* Kai-Xin Zhang,* Bin Wang,* Regis Doyonnas,* Manfredo Quadroni,* and John W. Schrader**

We purified from activated T lymphocytes a novel, highly conserved, 116-kDa, intracellular protein that occurred at high levels in the large, dividing cells of the thymus, was up-regulated when resting T or B lymphocytes or hemopoietic progenitors were activated, and was down-regulated when a monocyctic leukemia, M1, was induced to differentiate. Expression of the protein was highest in the thymus and spleen and lowest in tissues with a low proportion of dividing cells such as kidney or muscle, although expression was high in the brain. The protein was localized to the cytosol and was phosphorylated, which is consistent with a previous report that the Xenopus laevis ortholog was phosphorylated by a mitotically activated kinase (1). The cDNA was previously mischaracterized as encoding p137, a 137-kDa GPI-linked membrane protein (2). We propose that the authentic protein encoded by this cDNA be called cytoplasmic activation/proliferation-associated protein-1 (caprin-1), and show that it is the prototype of a novel family of proteins characterized by two novel protein domains, termed homology regions-1 and -2 (HR-1, HR-2). Although we have found evidence for caprins only in urochordates and vertebrates, two insect proteins exhibit well-conserved HR-1 domains. The HR-1 and HR-2 domains have no known function, although the HR-1 of caprin-1 appeared necessary for formation of multimeric complexes of caprin-1. Overexpression of a fusion protein of enhanced green fluorescent protein and caprin-1 induced a specific, dose-dependent suppression of the proliferation of NIH-3T3 cells, consistent with the notion that caprin-1 plays a role in cellular activation or proliferation. The Journal of Immunology, 2004, 172: 2389–2400.

The cellular elements of the immune system are generated continuously throughout adult life through tightly controlled cellular proliferation. The continuous generation of naive B or T lymphocytes depends on the extensive proliferation in the bone marrow or thymus of the precursors from which mature lymphocytes are selected. The ability of the immune system to respond to pathogens also depends upon the rapid proliferation of both Ag-specific T and B lymphocytes and of the progenitors of granulocytes, macrophages, and mast cells. Analysis of the signal transduction events induced by growth factors and of the genetic defects in leukemias and lymphomas has resulted in the identification of many of the key molecules involved in proliferation of lymphohemopoietic cells and their precursors. These include members of well-known protein families, such as the Ras superfamily, the mitogen-activated protein (MAP)3 kinase family, and the tyrosine kinases. However, it is unlikely that all of the proteins that participate in cellular growth and proliferation have been identified, and some of these are likely to lie within the ~40% of human proteins that have no known function or homology with known proteins (3), or the ~1800 known protein domains (4). In this study, we describe the identification of a novel, cytoplasmic phosphoprotein that was up-regulated when resting, G0 T or B lymphocytes were activated, and was present in the highest levels in the thymus and spleen.

We term this protein cytoplasmic activation/proliferation-associated protein-1 (caprin-1), and characterize it as the prototype of a novel family of proteins that is highly conserved throughout vertebrate evolution.

Materials and Methods
cDNA constructs and Abs
A cDNA-encoding human caprin-1 (BC001731) was obtained from Research Genetics (Huntsville, AL). Full-length, human caprin-1 or the indicated fragments were amplified by PCR and cloned into the mammalian expression vectors, pEGFP-C1 (Clontech Laboratories, Palo Alto, CA), pEBG (a gift from L. Zon, Dana-Farber Cancer Institute, Boston, MA), pIRES-2 (Clontech Laboratories), or the retroviral vector pMXP-PIC (a gift from A. Mui, Jack Bell Research Center, Vancouver General Hospital, Vancouver, British Columbia, Canada) to generate constructs encoding N-terminal enhanced green fluorescent protein (GFP) or GST fusion proteins, or a C-terminal hemagglutinin (HA) epitope-tagged version of caprin-1, respectively. The cDNA previously reported to encode p137 (2), as well as an antisera against a polypeptide encoded by part of this cDNA were the kind gifts of P. Luzio (University of Cambridge, Cambridge, U.K.). Affinity-purified rabbit Abs specific for the peptide, EKLMDLL DRHVEDGNVTVQHA, termed affinity-purified (AP) Abs, were prepared from hyperimmune serum.

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3 Abbreviations used in this paper: MAP, mitogen-activated protein; AP, affinity purified; BHP, Bombyx HR-1-containing protein; caprin, cytoplasmic activation/proliferation-associated protein; CRD, C1q-related domain; DHP, Drosophila HR-1-containing protein; ERK, extracellular signal-regulated kinase; EST, expressed sequence tag; GFP, enhanced green fluorescent protein; HA, hemagglutinin; HR, homologous region; PFA, paraformaldehyde; PTB, phosphotyrosine binding domain; SH2, Src homology 2; UTR, untranslated region; smgGDS, small G-protein guanine nucleotide dissociation stimulator.

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NOVEL PROTEIN UP-REGULATED IN DIVIDING CELLS AND BRAIN

cDNA consensus sequences for human, mouse, and *Xenopus laevis* caprin-1, and human caprin-2 were generated using a series of overlapping expressed sequence tag (EST) clones from the National Center for Biotechnology Information databases (www.ncbi.nlm.nih.gov). All sequences were covered by a minimum of three overlapping EST clones. If only two overlapping EST clones were present, the sequence was confirmed using human or mouse genomic sequence. Consensus sequences were assembled using Sequencher (Gene Codes, Ann Arbor, MI) and Macvector (Accelrys, San Diego, CA) software.

Clones used to generate the caprin-1 sequences included: for the human, BC001731, BG720819, BG724489, B1253821, AU162920, BE902810, AL040074, AU131547, BG252928, AU1322904, BG247359, AU141221, BG681409, B129287, AU119855, BG291262, BG633250 (EST: www.ncbi.nlm.nih.gov/entrez/query.fcgi), for the mouse, BE288344, AI11506, BF137301, X98571, B152150, B1437836, B1965135, BF467450, BF461675, AA606083, BE951949, AI140302, BF099994, AW913584, B1156548, AA183811, AI007156, BI695199, BF101269, BG070252, BG084803 (EST encoding alternative 5′ UTR: NM 016739); and for *X. laevis*, AUG72143, BG048625, BE599884, BG408527, BE491274, BG084081, BE679686, AW060719, AW200655, BG347661, BG630636, BG021521, BF614499, AW200458, BE491444, BG486636, BE491455, BG345580.

Clones used to generate the consensus sequence for human caprin-2 included: AY704490, AW922778, BG494982, AI117060, BE744846, AL536436, AA310016, AI092672, Z44678, AI439883, BG250521, AA627575, BG205252, BE155964, R59224, BF277469, BE378048, BG198633, BG201727, AL566335, AW022120, AI689001, BF589574, BG941654.

Consensus sequences were submitted to GenBank-third-party database. The accession numbers are BK001104 for human caprin-1, BK001105 for murine caprin-1, BK001106 for *X. laevis* caprin-1, and BK001103 for human caprin-2.

Signal peptide and protein domain searches were performed using the SignalP software (www.cbs.dtu.dk/services/SignalP) and the Prosite software (www.expasy.org), respectively. Dendrograms were generated using the Treetop software available at the Genebee website (www.genebee.msu.su).

Exon boundaries were derived by analysis of acceptor-donor sites in contiguous, human genomic sequence (National Center for Biotechnology Information) (AC090469, AC068306, and AL049652 for caprin-1, and AC010198 for caprin-2), and confirmed using the BLAT tool (5) (University of Santa Cruz, Santa Cruz, CA; www.genome.ucsc.edu). The Department of Energy Joint Genome Institute (JGI: www.jgi.doe.gov) provided databases of *Fugu rubripes* and *Ciona intestinalis* genomes.

**Culture.** Cells were cultured, as described previously (6, 7), and 10-cm dishes were transfected with 8 μg of DNA using lipofectamine 2000 reagent (Invitrogen, San Diego, CA), according to the manufacturer’s instructions. Primary T and B lymphoblasts were generated from mouse splenocytes by stimulation with Con A and IL-2 for 48 h, or an anti-CD3 mAb (2C11; BD PharMingen, San Diego, CA) and IL-2 for 3 days, or LPS for 4 days (7). Bone marrow cells from the posterior bone marrow of region (UTR), AF152163; hybrid mice were cultured in RPMI 1640 supplemented with 10% FCS and 4% WEHI-3B-conditioned medium as a source of IL-3, or 20% L cell-conditioned medium as a source of CSF-1. Differentiation of M1 leukemia cells was stimulated with murine rIL-6 (Intergen, Purchase, NY). Caco-2 cells were cultured at subconfluence in DMEM supplemented with 20% FBS, l-glutamine, and sodium pyruvate.

**Immunoblotting and immunoprecipitation**

Cells or tissues were lysed with buffer (7) containing 1% Nonidet P-40, or for multimerization/coprojection experiments 0.2% Triton X-100, and the lysates were normalized for total protein content. For immunoblotting, samples were run on SDS-PAGE, and the separated proteins were transferred to nitrocellulose and immunoblotted (7) with the indicated rabbit or mouse Abs. Primary Abs were detected with alkaline phosphatase or horse radish peroxidase (HRP) conjugated to goat anti-rabbit or goat anti-mouse Abs conjugated to AlexaFluor 594 (Molecular Probes, Eugene, OR).

**2P labeling of caprin-1**

Baf3 cells were incubated at 2.5 × 10^6 cells/ml in phosphate-free DMEM (Invitrogen) with 10% dialyzed FCS, saturating concentrations of murine IL-3 (7), and HEPES (10 mM) for 2 h. A total of 2 mCi of ^2P-labeled phosphoric acid (ICN Pharmaceuticals, Costa Mesa, CA) was added, and 1–6 h later caprin-1 was immunoprecipitated from cell lysates, and the precipitates were subjected to SDS-PAGE, autoradiography, and immunoblotting.

**Results**

**A 116-kDa cytoplasmic protein is up-regulated in proliferating T or B lymphoblasts**

In an attempt to determine whether the guanine-nucleotide exchange factor, small G-protein guanosine nucleotide dissociation stimulator (smdGDS) (9), was involved in the activation of T lymphocytes, we treated splenocytes with the T lymphocyte mitogen, Con A, and examined whether levels of smdGDS increased. Immunoblots were performed using AP Abs specific for the peptide sequence, EKLMDDLDRHVEDGNTVQHA, that corresponded to aa 360–380 of smdGDS. However, these AP Abs failed to recognize the expected 55-kDa splice form of smdGDS in lysates of T lymphoblasts (B. Grill and J. W. Schrader, unpublished observation) and instead revealed a 116-kDa protein (p116) that occurred at much higher levels in activated T lymphoblasts than in unstimulated splenocytes (Fig. 1, A and B). The lysates were normalized for total protein content before immunoblotting, the increase in cell size that occurred with T cell activation could not account for the observed increase in levels of p116. Similar results were obtained when T lymphocytes were specifically activated by ligation of CD3, a component of the TCR for Ag (data not shown). To determine whether increased levels of p116 were specific for T lymphocyte activation/proliferation, we stimulated splenocytes with a mitogen for B lymphocytes, LPS. We observed that AP Abs also detected increased levels of p116 in blast cells derived from B lymphocytes (Fig. 1B). These results indicated that p116 was up-regulated when resting T or B lymphocytes were activated into cell division, and suggested that this protein might be involved in cellular activation or proliferation.

We then investigated expression of p116 in immunoblots of a variety of lymphohemopoietic cell lines of both the lymphoid and myeloid lineages. We observed high levels of p116 in the B lymphoma WEHI-231, and also in R6X (IL-3-dependent mast cell/
FIGURE 1. Expression pattern and localization of p116. Whole cytoplasmic lysates (normalized for total protein content of splenocytes, T lymphoblasts, or B lymphoblasts from A, (C57BL6 × DBA)F1 hybrid (BDF1) mice, or B, BALB/c mice, were run on SDS-PAGE, and immunoblotted (IB) with AP Abs generated against a peptide from the guanine nucleotide exchange factor, smgGDS. Equivalency of loading was confirmed by reprobing blots with Abs against ERK (anti-ERK). C, Whole cell lysates from a variety of murine cell lines were immunoblotted with AP Abs. D, AP Abs were used to precipitate p116 (+) from Baf3 cells. E, Intracellular staining of splenocytes (thin line, AP; dotted line, secondary alone) or T lymphoblasts (thick line, AP; dashed line, secondary alone) from BDF1 mice assessed by flow cytometry. F, Murine bone marrow cells were cultured for 6 days with conditioned medium containing either IL-3 or CSF-1, as indicated. Shown are the profiles of the large blast cells (thick line, AP; dashed line, secondary alone). G, Staining with AP Abs of live Baf3 cells, or Baf3 cells fixed with PFA and permeabilized with saponin (thin line, secondary alone; thick line, AP; dashed line, AP + specific peptide; dotted line, AP + nonspecific peptide). H, Cytospun Baf3 cells were fixed in PFA, methanol permeabilized, and stained with AP Abs with or without specific peptide. AP binding is shown in red, and 4',6'-diamidino-2-phenylindole staining of the nuclei in blue. I, Immunoblot with AP Abs of indicated tissues from an adult BALB/c mouse.
megakaryocytic cells), the IL-3-dependent cell line Ba/F3 (Fig. 1C), and in M1 monocytic leukemia cells (Fig. 1D). Immunoblots of lymphoblasts (Fig. 1A), or Ba/F3 cells (Fig. 1C), or of immunoprecipitates made with AP Abs from lysates of Ba/F3 cells (Fig. 1D) demonstrated that AP Abs detected only p116 in these cells. Therefore, we were able to use AP Abs to compare levels of expression of p116 in individual cells using flow cytometry, and to investigate its subcellular localization using immunofluorescence. Flow cytometry showed that T lymphoblasts (Fig. 1E) or B lymphoblasts (data not shown) exhibited ~15-fold greater staining with AP Abs than resting lymphocytes, consistent with the results of immunoblotting (Fig. 1A). Likewise, blast cells (corresponding to immature, proliferating hemopoietic cells) that were generated from murine bone marrow by culture with conditioned medium containing either IL-3 or CSF-1 stained strongly with AP Abs (Fig. 1F).

Unfixed, live Ba/F3 cells did not react with AP Abs, while cells that were fixed and permeabilized did (Fig. 1G), indicating that p116 was not expressed on the cell surface, but was present inside the cell. The specificity of this staining was confirmed by the demonstration that it was inhibited by competition with the specific peptide targeted by AP Abs, but not with an irrelevant peptide (Fig. 1G). We used AP Abs and immunofluorescence to examine the intracellular localization of p116 in Ba/F3 cells. We observed that the fluorescence (shown to be specific by inhibition by competing peptide) was evenly distributed throughout the cytoplasm and excluded from the nucleus (Fig. 1H).

Expression of p116 in tissues and nonlymphohemopoietic cell lines

We observed similar, high levels of p116 in AP immunoblots of every dividing cell examined, including cell lines of epithelial origin (scp-2 murine mammary cells, HEK 293 human embryonic kidney cells) and mesenchymal origin (3T3 fibroblasts, L929 endothelial cells, C2 myoblasts) (Fig. 1C and data not shown). Levels of p116 were highest in the thymus and spleen, whereas kidney, muscle, or liver showed very little immunoreactivity (Fig. 1J). This pattern of expression was consistent with the notion that levels of p116 correlated with the frequency of dividing cells, although one notable exception to this generalization was the brain, which exhibited high levels of p116. However, many other proteins associated with cell division, such as proteins in the Ras/extracellular signal-regulated kinase (ERK) MAP kinase pathway, and Src family kinases are also highly expressed in the brain, where they are involved in the transmission of signals from extracellular stimuli (10). Thus, the pattern of expression of this 116-kDa protein was consistent with a role, whether positive or negative, in cellular activation and proliferation. We gave the protein the operational name of cytoplasmic activation/proliferation-associated protein-1, caprin-1.

Caprin-1 is expressed at high levels in dividing thymocytes

All the subpopulations of thymocytes, including both CD4 and CD8 single-positive cells, CD4 and CD8 double-positive, and double-negative cells, stained with the AP Abs (Fig. 2A and data not shown). However, there was clear heterogeneity in the levels of staining, with a notable subpopulation of more brightly staining cells (Fig. 2A). Multiparameter analysis indicated that this population of brightly staining cells was made up of the large, dividing blast cells (Fig. 2B). The mean fluorescence intensity of the large, dividing blast cells was 3-fold higher than that of the small, nondividing thymocytes that are more differentiated.

Structure of caprin-1

We used AP Abs to immunoprecipitate caprin-1 from lysates of T lymphoblasts. Coomassie staining of the immunoprecipitate after SDS-PAGE revealed the expected 116-kDa band, which was not present in a control preparation made in parallel using Igs from unimmunized rabbits (data not shown). The 116-kDa band was excised and analyzed by mass spectrometry. The two peptide sequences we obtained (LNQDQLDAVSK and YEVTNNLEFAK) were both present in the sequence of a human protein termed p137 (2) or GPI-anchored membrane protein (11). However, analysis of ESTs and human draft genomic sequence (National Center for Biotechnology Information) indicated that the published data contained a series of important errors. First, a single base deletion in the p137 cDNA had resulted in a frame shift. As a result, the C terminus of both the deduced protein and the recombinant proteins used in this study lacked 80 aa of the authentic protein, which were replaced by an artificial sequence. This artificial region of 74 aa included the presumptive site for the GPI linkage reported by these authors (2), which thus does not exist in the authentic protein. Another error at the 5’ end of the p137 cDNA sequence introduced an artificial stop codon. This resulted in misidentification of the initiating methionine and the truncation of the first 53 aa from the authentic N terminus. There was no evidence of a signal peptide in the authentic protein sequence. We assembled EST sequences and translated them to generate consensus sequences for caprin-1 in the human, mouse, and X. laevis (consensus sequences compiled on GenBank third-party annotation database; accession numbers: BK001104, BK001105, BK001106) (Fig. 3A). There was a striking degree of amino acid conservation, with the human and mouse sequences being 97% conserved (96% identical), and the human and X. laevis 80% conserved (68% identical).

During the course of our study, a human cDNA (BC 001731) and a model human mRNA (XM_011991) and protein (XP_011991) corresponding to the protein that we deduced were added to the public database (National Center for Biotechnology Information). The 709 aa predicted a protein of 78.4 kDa, far smaller than the apparent size (116 kDa) of the native protein we had purified and observed in immunoblots. However, a fusion protein of GST and the protein encoded by the cDNA BC 001731, which had a predicted M, of ~106 K, exhibited anomalously slow migration on SDS-PAGE with an apparent M, of 146 ± 3 K (Fig. 4A).
This aberrant migration was not due to posttranslational modification specific for mammalian cells because it occurred when fusion proteins were expressed in either mammalian or bacterial cells (Fig. 3B). The ability of AP Abs to recognize nanogram quantities of this fusion protein (Fig. 3C) confirmed that the protein encoded by the cDNA BC 001731 was identical with p116/caprin-1.
(Fig. 3C). Furthermore, when we accounted for the ~30 K of relative mobility contributed by GST, we calculated the relative mobility of recombinant caprin-1 as 116 ± 3 K, which was approximately the same as that of endogenous caprin-1.

It was not clear why the AP Abs raised against the peptide from smgGDS were able to bind caprin-1, because the amino acid sequence of caprin-1 did not contain any sequences of 4–6 contiguous aa that were identical with those present in the smgGDS peptide. Nonetheless, the epitope recognized by the peptide Abs is likely to be linear, as it is recognized both on the native molecule and in immunoblots of what are likely to be extensively denatured material.

In reciprocal experiments, a rabbit antiserum raised against a rGST-fusion protein that included aa 189–629 of the protein encoded by the BC001731 cDNA recognized the 116-kDa band that was immunoprecipitated from Balb/3 cells by AP Abs (Fig. 3D). This antiserum also detected a 116-kDa band that was up-regulated in T or B lymphoblasts (Fig. 3E), and was expressed in various tissues (Fig. 3F) at the same relative levels as the 116-kDa band detected by the AP Abs. Thus, the 116-kDa protein we termed caprin-1 was identical with the protein encoded by the cDNA BC 001731, and represented the authentic version of the protein that was encoded by the cDNA previously described as encoding GPI-anchored membrane protein p137 (2).

It was conceivable that some caprin-1 was present on the cell surface in a form that was not recognized by the AP Abs. Therefore, we expressed full-length caprin-1 (cDNA BC001731) fused at the N terminus with GFP (GFP-caprin-1) or at the C terminus with a HA epitope tag (caprin-1-HA) and examined Bosc23 cells or 3T3 cells by fluorescence microscopy (Fig. 3G) or immunofluorescence (Fig. 3H), respectively. In neither case could we detect localization at the membrane, and the tagged recombinant caprin-1 exhibited the same cytosolic distribution and exclusion from the nucleus as was seen with staining of endogenous caprin-1 using exon 1 and has an in-frame stop codon, 5’ to the initiating methionine (Fig. 4C). Examination of ESTs and RT-PCR (data not shown) indicate that both UTR are conserved in mouse and human. There was also EST evidence for alternative splicing that would result in an alternate C terminus encoded by exon 18’ (consensus sequence compiled on GenBank third-party annotation database, accession number: BK001101). This alternatively spliced message would encode a protein in which the 21 C-terminal amino acids of caprin-1 were replaced with five new residues, NILWW, which are identical in mouse (BG708972) and human (BG708972). Exon 18’ also includes a long 3’ UTR that exhibits a high level of conservation between mouse and human. The caprin-1 cDNA that includes exons 18 and 19 has a long 1.1-kb 3’ UTR (Fig. 4C) that is also highly conserved in the mouse and human (overall identity 87%), as well as in the chicken. A smaller 204-bp region is 88% identical in human and X. laevis. The remarkable conservation of these 3’ UTR suggests they are involved in important functions, which may include posttranscriptional regulation of levels of caprin-1 or subcellular localization of its mRNA (12).

Homologous region-1 (HR-1), a novel protein domain highly conserved in vertebrates and insects

We found no homologues of caprin-1 in yeast or Caenorhabditis elegans, but the Drosophila melanogaster genome encoded a novel hypothetical protein of 961 aa (AAG22572), which included a region with 32% identity and 52% similarity with a region near the N terminus of human caprin-1. We termed this HR-1 and the predicted D. melanogaster protein, Drosophil a HR-1-containing protein (DHP). We also identified Bombyx mori ESTs (AV398342, AV398325, AV398082, and AU003960), which encoded part of a third novel protein that exhibits a well-conserved HR-1 domain (38% identity and 61% conservation with the HR-1 domain of human caprin-1) (Fig. 4A), and which we termed Bombyx HR-1-containing protein (BHP). DHP, BHP, and human caprin-1 exhibited no homology in any region other than HR-1. Interestingly, the two insect HR-1 domains were no more similar to each other than to the HR-1 domain of human caprin-1 (Fig. 4A). The level of sequence conservation of the HR-1 in these three distinct proteins is remarkable, and suggests strongly that HR-1 represents a novel, independently folding protein domain that is likely to have a conserved function.

Caprin-2

We also found HR-1 in a paralog of caprin-1 that we identified in the EST and cDNA databases (National Center for Biot echnology Information) and termed caprin-2. Fig. 4B shows a consensus sequence for caprin-2 (accession number: BK001103). The HR-1 domain of human caprin-2 was 73% conserved (51% identical) compared with that of human caprin-1. Caprin-2 also exhibited a second region of homology, that we termed “homologous region-2” (HR-2), and exhibited 54% conservation (36% identity) with 334 aa near the C terminus of human caprin-1. Well conserved orthologs of caprin-2 were identified in vertebrates including mammals, amphibians, and Danio rerio and Fugu rubripes. HR-2 exhibited no similarities with known domains, or structural motifs suggestive of function, and occurs only in caprin-1 and -2. Caprin-2 differed from caprin-1 in the presence of a third domain
at the C terminus (Fig. 4, B and C) that was highly conserved in human and mouse caprin-2 (98% identity) and was homologous to the globular domain of C1q. We termed this the C1q-related domain (CRD) (Fig. 4). The C1q domain and the related region in TNF-α are involved in multimerization (13), and it is possible the CRD of caprin-2 may have a similar function. We used RT-PCR with primers spanning HR-2 and the CRD to demonstrate that mature, spliced caprin-2 transcripts that included the CRD were

**FIGURE 4.** The caprin-1 and insect HR-1 domains, and the human caprin-1 and caprin-2 proteins and genes. A, Alignment (Clustal-W) of the HR-1 domains from BHP, DHP, and human caprin-1, and a dendrogram of these plus the HR-1 domain of human caprin-2. B, Alignment of human caprin-1 and caprin-2 and their HR-1 (red line) and HR-2 (blue line) domains, and the CRD (green line) of caprin-2. The first three N-terminal methionine residues of caprin-2 are highlighted in red. C, Caprin-1 and caprin-2 genes with the exons encoding HR-1, HR-2, and the CRD indicated. Also shown are the alternative 5' and 3' UTR of caprin-1.
expressed in murine spleen (data not shown). The CRD of caprin-2 is annotated in the databases as a distinct hypothetical protein, FLJ22569, with a corresponding provisional gene, FLJ22569.

Analyses of the EST, cDNA, and serial analysis of gene expression databases (National Center for Biotechnology Information) confirmed that, as with caprin-1, caprin-2 mRNA is present in hematopoietic cells, including erythroid progenitor cells (AY074491, AY074490), and in the brain. Analyses of the EST and cDNA databases predict that in humans, alternative splicing generates two proteins with predicted sizes of 126 and 117 kDa and with the respective N-termini MEQV and MKS AK (Fig. 4B, highlighted in red). Neither of these proteins includes a signal peptide. There is EST evidence for an X. laevis protein that starts at the intervening methionine in a conserved MQVLF motif that is also indicated in Fig. 4B.

The National Center for Biotechnology Information databases also provide evidence for multiple splice variants of caprin-2 involving differential use of internal exons (BG944982, AL536436, BT69244, AA187575, BE155973, and AY074490). Human caprin-2 has a polymorphism (K/R) at residue 237. Differential splicing can result in the addition of an alanine at residue 823 (AA134604, BE155964, BG202526; consensus sequence compiled on GenBank third-party annotation database, accession number: BK001102). Similar differential splicing of this exon leading to the addition of a single amino acid also occurs in the mouse and chicken.

Genomic organization

The HRs of the human caprin-1 and caprin-2 genes exhibit a similar organization of exons and introns (Fig. 4C). The number and size of exons encoding HR-1 (6 exons) and HR-2 (9 exons) are the same in both genes (Fig. 4C), consistent with their origin from gene duplication. The CRD of caprin-2 is encoded by a single exon. There is conservation of synten for both caprin-1 and caprin-2 loci. In the murine and F. rubripes genomes, orthologs of the human genes for LMO-2 (Lim domain only-2 protein), caprin-1, and FLJ10778 (annotated as an acetyltransferase) are linked in the same order and orientation as in the human. Both the human caprin-2 gene and its F. rubripes ortholog are adjacent to orthologous genes encoding the Ras-related nuclear protein-binding protein 8. The most primitive organism in which we have evidence for a caprin is the urochordate, C. intestinalis. Its genome encodes a single, well-conserved caprin gene that is likely to be the ancestral gene of both caprin-1 and -2. The genome of C. intestinalis also includes a gene encoding a cerebellin-like protein (ci0100144094), which is closely related to the C-terminal 130-aa residues of the CRD of human caprin-2 (38% identity, 53% conserved). It is possible that the vertebrate caprin-2 gene resulted from fusion of part of this cerebellin-like gene with a duplication of the ancestral urochordate caprin gene.

Caprin-1 is a phosphoprotein that exists in a multiprotein complex X. laevis. Caprin-1 was previously identified in a screen for cDNA-encoding proteins that were phosphorylated by mitotically activated kinases present in an extract of fertilized oocytes (1). However, because of its previous misidentification as a GPI-anchored membrane protein, it was concluded that its phosphorylation by these kinases was an artifact of the screening strategy and was not physiologically relevant. We used AP Abs to immunoprecipitate caprin-1 from Baf3 cells labeled for 1–6 h with 32P-labeled orthophosphate. In three independent experiments, autoradiographs demonstrated a major phosphorylated species of $M_t$ 116 K, together with two doublets of $M_t$ ~66 K and ~33 K (Fig. 5). The upper band ($M_t$ 116 K) was recognized by anti-recombinant caprin-1 Abs, confirming its identity as phosphorylated caprin-1. Because the anti-recombinant caprin-1 Abs were raised against the entire caprin-1 protein with the exception of the first 53 and the last 80 aa, the fact that these Abs did not detect bands corresponding to the 66 K or 33 K doublets indicates that these doublets were unlikely to be proteolytic fragments of caprin-1. Similar $M_t$ ~33 K and 66 K 32P-labeled doublets coprecipitated with GST-caprin-1, but not with GST alone when these proteins were expressed in 32P-orthophosphate-labeled cells (data not shown).

Both PhosphoBase (www.cbs.dtu.dk) and Prosite (www.expasy.org) identified a series of potential serine/threonine phosphorylation sites in caprin-1. The kinases for which potential consensus phosphorylation sites were found included: calcium/calmodulin-dependent kinase II, casein kinase I and II, glycerokinase threonine kinase-3, and protein kinases A and C. When we examined evolutionary conservation of these potential phosphorylation sites and the neighboring amino acids, we found several residues (serine 179, 200, 287, 437, 439, and 663; threonine 126, 148, and 229) that were conserved in human and X. laevis caprin-1. Interestingly, several of these potentially phosphorylated residues, along with the neighboring amino acids, were also conserved in caprin-2. These residues included serine 200 and 663, and threonine 229. Although neither program predicted phosphorylation at conserved tyrosine residues, there was evidence for binding sites for phosphotyrosine binding domains (PTB) and Src homology 2 (SH2) domains, suggesting these tyrosine residues may be phosphorylated. We found four tyrosine residues in the HR-1 and -2 domains that met the consensus sequence for SH2 binding sites, Y-X-X-hydrophobic (14). Three of these SH2 binding sites were conserved between human and X. laevis caprin-1 (tyrosine 99, 207, 230) and were all located in the HR-1 domain. The tyrosine residue of the fourth SH2 binding site was also conserved; however, the hydrophobic amino acid required for the consensus was mutated in X. laevis. Two of these potential SH2 sites were conserved in caprin-2 (tyrosine 99 and 230). There was also evidence for two potential PTB binding sites in the HR-2 domain that corresponded to the consensus N-X-X-Y (14). One of these potential PTB binding sites (tyrosine 651) had both the tyrosine and N-terminal asparagine residue conserved in both human and X. laevis caprin-1, as well as caprin-2.

In other experiments, we explored the possibility that caprin-1 existed in multimeric complexes. We observed that immunoprecipitation of a GFP-caprin-1 fusion protein using Abs to GFP resulted in coprecipitation of coexpressed, HA-tagged caprin-1 (Fig. 6, B and C). Precipitation of a GFP fusion of a deletion mutant (HR-1) that corresponded essentially to HR-1 also resulted in coprecipitation of HA-tagged caprin-1 (Fig. 6). In contrast, only
small amounts of HA-tagged caprin-1 were coprecipitated with a fusion protein of GFP and essentially HR-2. These results suggest that the HR-1 region is important for formation of a complex containing two or more caprin-1 molecules.

**Levels of caprin-1 decrease when cells are deprived of growth factors or are induced to differentiate**

To investigate changes in levels of caprin-1 when cells ceased to divide, we deprived IL-3-dependent Ba/F3 cells of IL-3. We observed a marked decrease in the levels of caprin-1 relative to total proteins (Fig. 7A). Similarly, serum-dependent NIH-3T3 cells that were cultured in the absence of serum and ceased to proliferate exhibited a 2-fold reduction in caprin-1 expression, as assessed by immunoblotting of equivalent amounts of total cellular protein with anti-recombinant caprin-1 Abs (Fig. 7C), or by flow cytometry and staining with AP Abs (Fig. 7D). In both cases, levels of control proteins, the ERK MAP kinases were unchanged. NIH-3T3 cells also exhibited a 2-fold reduction in caprin-1 expression levels when they were cultured at confluence and ceased proliferating (Fig. 7C).

We used flow cytometry and AP Abs to determine whether caprin-1 was differentially expressed at different points in the cell cycle (Fig. 7E). Although there was a slight increase in caprin-1 levels in cells in S and G2/M phase, this was likely to be explained by the increases in cell size in S and G2/M phase cells, as there were similar increases in levels of ERK-1 and -2 (Fig. 7E). We concluded that in continuously cycling cells, caprin-1 is expressed at equivalently high levels in the G1, S, and G2/M phases of the cell cycle.

We also treated the mouse leukemic cell line M1 with IL-6, which induces it to differentiate into adherent, nonproliferating macrophages. We observed that cessation of growth and differentiation was accompanied by decreased levels of caprin-1 (Fig. 7B).

**Overexpression of GFP-caprin-1 results in specific, dose-dependent inhibition of cell division**

We used flow cytometry to determine whether overexpression of caprin-1 affected proliferation of NIH-3T3 cells. We observed that overexpression of GFP-caprin-1 resulted in dose-dependent decreases in cell numbers relative to those of cells expressing equimolar amounts of GFP alone (data not shown). To determine whether this was due to interference with cell division, we transfected NIH-3T3 cells with either GFP-caprin-1 or GFP alone, and labeled them with a fluorescent dye, PKH26, that associates permanently with the plasma membrane and is diluted 2-fold each time a cell divides. Using two-color flow cytometry, we compared the number of divisions undergone by cells expressing various levels of GFP-caprin-1, or equimolar levels of the control protein GFP. As shown in Fig. 8, cells expressing mean levels of GFP-caprin-1 that were 3-fold in excess of endogenous levels of caprin-1 (mean level, L) exhibited significantly longer doubling times than control, untransfected cells in the same culture, or cells expressing GFP alone. This effect was dose dependent, and cells expressing GFP-caprin-1 at mean levels that were in 12-fold (mean level, M) or 48-fold (mean level, H) excess over endogenous levels of caprin-1 divided infrequently or failed to divide at all (Fig. 8). We saw no evidence that overexpression of GFP-caprin-1 led to increased cell death from apoptosis (B. Grill and J. W. Schrader, unpublished observations).

**Discussion**

Levels of caprin-1 in cells of the lymphohemopoietic system correlated tightly with their proliferative status. Activation of T or B lymphocytes to proliferating blast cells was associated with 15-fold increases in the amount of caprin-1 per cell (Fig. 1). Hemopoietic progenitors proliferating in response to IL-3 or CSF-1 also expressed high levels of caprin-1 (Fig. 1F). Similarly, when serum-starved NIH-3T3 cells were stimulated with serum, there was a 2-fold increase in levels of caprin-1 (Fig. 7, C and D). In contrast, levels of caprin-1 decreased when proliferating, IL-3-dependent cells were deprived of IL-3 (Fig. 7A), or serum-dependent NIH-3T3 cells were cultured in the absence of serum to halt proliferation (Fig. 7C). Similarly, when M1 leukemia cells were induced to differentiate to nondividing, adherent macrophages, caprin-1 levels decreased significantly (Fig. 7B).

The correlation of proliferation with increased expression of caprin-1 was also seen when levels of caprin-1 in different tissues were compared. Thus, the tissue with the highest level of expression of caprin-1 was the thymus, a site of continuous cellular proliferation. Significantly, while all thymocytes contained caprin-1, the cells with the highest levels were the large, dividing blast cells (Fig. 2). In contrast, caprin-1 levels were low in tissues such as the kidney or muscle that have a low proportion of dividing cells. The one exception was the adult brain, which expressed significant levels of caprin-1 despite having a low percentage of dividing cells. It is possible that caprin-1 resembles components of the Ras and MAP kinase pathways, which are critical for cell growth, but also have key functions in nondividing cells in the brain (10).

We provide evidence in this study for a new gene family, the caprins, and demonstrate that caprin-1 is a 116-kDa, cytoplasmic phosphoprotein that was encoded by a cDNA that was previously erroneously reported to encode a 137-kDa GPI-linked membrane protein (2). The correction of such errors in the literature and databases derived from the literature is of particular importance, as databases are finding extensive use in predicting the function of homologues or of
binding proteins identified in global screens. For example, Kirschner and colleagues (1) twice isolated caprin-1 cDNA clones in a screen for proteins that were phosphorylated by mitotically activated kinases. However, the misidentification of caprin-1 as a GPI-linked membrane protein led them to discard this finding as an artifact. Our data showing that caprin-1 is in fact a cytosolic phosphoprotein, which varies in levels with the state of cellular activation, suggest that the observation of Kirschner and colleagues was valid and provides clues to the function of caprin-1. Further support for the observation that caprin-1 is a phosphoprotein was provided by identification of potential phosphorylation sites in caprin-1, as well as potential sites for binding of SH2 and PTB domains that were conserved in caprin-1 and -2.
The misidentification of the protein encoded by the caprin-1 gene as a 137-kDa GPI-linked membrane protein was not based upon expression of the cDNA in mammalian cells and investigation of the localization of the protein, but on two lines of indirect evidence. First, an in vitro translation product of p137 was incorporated into liposomes (2). However, due to a 1-base deletion, the cDNA used encoded an artificial sequence of 74 aa at the C terminus, which contained the postulated site for GPI linkage and may have accounted for the anomalous partitioning of the protein. Second, their experiments that demonstrated the GPI linkage of p137 used Abs raised to products expressed from parts of their cDNA sequence to immunoprecipitate a membrane protein. In that we failed to see endogenous caprin-1 or recombinant GFP-caprin-1 or caprin-1-HA on the surface of Caco-2 cells or any other cell type tested, we conclude that the 137-kDa GPI-linked membrane protein they studied was not encoded by the caprin-1 cDNA, but instead represented an unrelated protein that serendipitously lead to a reassessment of the significance of the report that the X. laevis ortholog of caprin-1 was twice identified in a screen for proteins that were phosphorylated by a mitotically activated kinase (1). Our data extend this observation by tightly correlating cellular activation and proliferation with increased levels of expression of caprin-1 and by demonstrating that caprin-1 is a phosphoprotein that interacts with other phosphoproteins.

We observed that the increase in levels of caprin-1 in actively dividing cells as compared with G₀ cells occurred in all phases of the cell cycle. A similar increase in levels of a protein in all phases of the cell cycle in a population of dividing cells has been reported for multiple proteins that include transcription factors (15, 16) and DNA topoisomerase II (17), as well as proteins involved in general metabolism (18). The correlation of caprin-1 expression with activation and proliferation does not demonstrate that its role in cellular activation/proliferation is a positive one, as negative regulators are up-regulated when T lymphocytes leave G₀ and begin to cycle (19, 20). However, in ongoing experiments, we have targeted the caprin-1 gene in the chicken B cell line, DT40, and observed that cells that are homozygous null at both caprin-1 alleles exhibit defects in cellular proliferation (B. Wang and J. W. Schrader, unpublished observation). Thus, caprin-1 is essential for proliferation of B lymphocytes and is likely to have a positive role in cellular proliferation. Our observation of a dose-dependent inhibition of

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Overexpression of GFP-caprin-1 blocks division of 3T3 cells. The 3T3 cells were transiently transfected with pEGFP-C1 encoding either GFP alone or GFP-caprin-1, and at 24 h were labeled with the lipophilic dye, PKH26. A, Shows on a logarithmic scale the expression of GFP-caprin-1 in three gates representing low (L), medium (M), and high (H) expression. B, Shows in each of the three gates (L, M, and H) of GFP-caprin-1 expression the ratio of the levels of GFP-caprin-1 to endogenous caprin-1. The ratios were determined by using staining with anti-caprin-1 Abs and flow cytometry to compare levels of endogenous caprin-1 in untransfected cells with total levels of caprin-1 in GFP-caprin-1 transfected cells. The gates L, M, and H have mean levels of expression of GFP-caprin-1 that are 3-, 12-, and 48-fold, respectively, above that of endogenous caprin-1. C, Shows levels of PKH26 dye fluorescence, immediately after labeling (thin line), or 3 days later (dashed line), of cells with levels of GFP-caprin-1 corresponding to those in each of the gates specified in A. D, Shows the number of cell divisions over 3 days of cells expressing the same three mean levels of GFP-caprin-1 or of cells expressing equimolar levels of GFP, or of untransfected 3T3 cells growing in the same culture.
proliferation when GFP-caprin-1 was overexpressed in NIH-3T3 cells (Fig. 8) is consistent with a role for caprin-1 in the assembly of a critical complex of proteins required for cellular proliferation. This notion is consistent with our evidence that caprin-1 exists in multimeric complexes that include unidentified phosphoproteins (Fig. 5). By titrating out its binding partners, overexpression of GFP-caprin-1 could lead to a decrease in the levels of functional complexes. This would result in a loss of complex function and inhibition of proliferation. However, it is also conceivable that the presence of GFP at the N terminus of GFP-caprin-1 may interfere with its function by creating a dominant inhibitory protein, thus resulting in a loss of caprin-1 function and inhibition of cellular proliferation.

That the thymus is the major site of expression of caprin-1 is consistent with a role for caprin-1 in cellular activation/proliferation. Moreover, in that there is considerable information on the role of signaling pathways in regulation of aspects of growth and survival in various stages of intrathymic differentiation (21–26), future experiments on the effects of tissue-specific ablation of caprin-1 genes in the thymus, or at particular stages of thymocyte development should shed light on its function in this tissue and provide further clues to the molecular mechanisms through which this intriguing molecule acts.

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