BI-RG-587, many HIV-1–infected cells stained blackish-purple. In the presence of BI-RG-587, no HIV-1–infected cells were seen. These results (*15*) were significant in that these isolates have never been adapted to grow in cells other than peripheral blood mononuclear cells (PBMC). They were tested in these experiments after the third PBMC passage.

We have described a novel nonnucleoside inhibitor of HIV-1 RT. BI-RG-587 acts as a noncompetitive enzyme inhibitor with exquisite specificity against HIV-1 RT. BI-RG-587 has potent antiviral activity against HIV-1 in vitro with extremely low cytotoxicity in uninfected human cells. Antiviral activity has been demonstrated against HIV-1 isolates from patients receiving AZT therapy. Like BI-RG-587, compounds reported by Pauwels *et al.* (*16*) have specificity for HIV-1 but not HIV-2. BI-RG-587 has shown no cytotoxic effects on human bone marrow colonies including erythroid burst-forming units and colony-forming units of granulocyte, erythroid, macrophage megakaryocyte and granulocyte macrophage at concentrations up to 37,500 nM (*17*). In cynomolgus monkeys, plasma levels remained between 35 and 140 times the IC_{50} (cell culture) during an 8-hour period after a single oral dose of 20 mg per kilogram of body weight. In chimpanzees, plasma levels of over 600 times the IC_{50} were observed after a single similar dose. In rodent and primate, tissue distribution studies after oral administration indicate a plasma:brain ratio of 0.8 to 1.0. Taken together, these results are encouraging for the development of BI-RG-587 as an antiviral for the treatment of HIV-1 infection in humans. The nonnucleoside nature of this compound may circumvent the associated toxicities of nucleoside chain terminators. Subsequent reports from our laboratories will delineate structure-activity relationships of dipyridodiazepinone inhibitors of RT, their metabolites and pharmacokinetics, effects on human bone marrow progenitors, and mechanism of action as RT inhibitors.

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gCap39, a Calcium Ion– and Polyphosphoinositide-Regulated Actin Capping Protein

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The polymerization of actin filaments is involved in growth, movement, and cell division. It has been shown that actin polymerization is controlled by gelsolin, whose interactions with actin are activated by calcium ion (Ca^{2+}) and inhibited by membrane polyphosphoinositides (PPI). A smaller Ca^{2+}- and PPI-regulated protein, gCap39, which has 49% sequence identity with gelsolin, has been identified by cDNA cloning and protein purification. Like gelsolin, gCap39 binds to the fast-growing (+) end of actin filaments. However, gCap39 does not sever actin filaments and can respond to Ca^{2+} and PPI transients independently, under conditions in which gelsolin is ineffective. The coexistence of gCap39 with gelsolin should allow precise regulation of actin assembly at the leading edge of the cell.

AGONIST STIMULATION INDUCES rapid actin polymerization in the cortical cytoplasm, a process that can be explained by an increase in the amount of polymerization-competent actin molecules and an increase in the number of actin-nucleating sites (*1*). The latter could be achieved rapidly by uncapping of existing actin oligomers that were blocked at the (+) ends by end-capping proteins. It has been suggested that membrane polyphosphoinositides promote nucleated actin filament growth by coordinated regulation of profilin (*2*), a protein that sequesters actin monomer, and gelsolin (*3*), a protein that caps, severs, and nucleates actin filament (*4*). A family of gelsolin-like, actin filament–severing proteins has been identified, and they have a highly conserved and internally repeated primary sequence (*5*). These include vertebrate villin (*6*), which is similar in size to gelsolin (*7–9*) (80 to 90 kD), and *Dictyostelium* severin (*10*) and *Physarum* fragmin (*11*), which are half as large and resemble the NH_2-terminal half of gelsolin. We report here the identification of a member of this family, gCap39, which caps actin filaments but does not sever them and dissociates from filament ends readily, either by decreasing Ca^{2+} to submicromolar concentrations or by increasing phosphatidylinositol 4,5-bisphosphate (PIP_2) without necessarily lowering Ca^{2+} concentration. Since gelsolin uncapping requires both an increase in PIP_2 and a decrease in Ca^{2+} concentration, gCap39 can generate actin nuclei by dissociating from filament ends under conditions in which gelsolin and other capping proteins remain associated.

The gCap39 cDNA was isolated during

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attempts to identify additional gelsolin-like proteins by cDNA cloning. We screened a mouse kidney cDNA library with a gelsolin NH_2-terminal cDNA probe (*7*), and clones that hybridized weakly were then sized and sequenced. Among these, clone C15 has a 1.2-kb insert that contains a single open reading frame starting with an ATG codon at base 20 and ending with a stop codon at base 1054 (*12*). The open reading frame (Fig. 1) encodes a protein of 351 amino acids, which is half as large as gelsolin. It shows extensive similarity to all known gelsolin-like proteins and has 49% sequence identity with the NH_2-terminal half of gelsolin. We named this protein gCap39 because of its molecular similarity to gelsolin, deduced mass, and actin-binding properties.

Northern blot analysis with the C15 cDNA probe showed that the hybridizing mRNA is about 1.2 kb long, similar in size to the cDNA insert (Fig. 2A). The mRNA is present in a large variety of tissues and is particularly abundant in kidney and lung.

The gCap39 protein was identified (independently of the cDNA cloning experiments) in macrophage cell lysate and culture medium as the predominant protein (40 kD), which binds to phenyl-Sepharose in a Ca^{2+}-dependent manner (Fig. 2B). Its identity with gCap39 cDNA was established by microsequencing of 13 of its peptide fragments (*13*). Although hydrophobic affinity chromatography has been used to isolate Ca^{2+}-binding proteins from liver and brain (*14*), gCap39 was not detected from these sources because they have very low gCap39 expression (Fig. 2A), in contrast to macrophages, which are rich in gCap39 (*13*). The presence of gCap39 in macrophage cell lysate and culture medium suggests that it is a cytoplasmic as well as a secreted protein. A precedent for this unusual dual existence has been established for gelsolin (*15*). The 5′ end sequence of C15 does not have the characteristics of a cleaved signal peptide; however, preliminary evidence indicates that the same cDNA specifies both the secreted and intracellular forms (*13*).

The actin-binding properties of gCap39 were examined by the pyrene-actin depolymerization assay (*16*). Capping of the (+) ends reduces the rate of actin depolymerization after dilution, whereas severing and capping increase the rate by generating large numbers of filaments that can depolymerize from the (−) ends. gCap39 decreased the depolymerization of actin filaments (Fig. 3A, curves b, c, and d) in the presence of Ca^{2+}, whereas gelsolin had the opposite effect. Therefore, gCap39 blocks filament ends and does not sever filaments. The binding affinity of gCap39 for actin filament ends, estimated from the depolymerization curves, was approximately 1.7 nM^{-1} in Ca^{2+}. EGTA inhibited capping (Fig. 3A, curve d_e), and removed gCap39 from ends of filaments (Fig. 3B, curve b). Within the gelsolin family, villin (*17*) can be uncapped by EGTA, but gelsolin (*18*) and severin (*19*) cannot.

Phosphatidylinositol 4,5-bisphosphate $(PIP)_2$ inhibited filament capping (Fig. 3A, curve d_p) and half-maximal inhibition of 6.8 nM gCap39 was observed at 100 nM PIP_2 (Fig. 3C). Although only submicromolar PIP_2 was used in the capping assay, the effective PIP_2/gCap39 ratio (29:1) is similar to that required for inhibition of severing by gelsolin (*3*) and severin (*20*) (39:1 and 32:1, respectively) determined at higher concentrations of PIP_2. This implies that gCap39 binds to PIP_2 with high affinity (K_d in the submicromolar range), which is comparable to that of human platelet profilin determined directly from binding studies (*21*). No inhibition was observed with 50 μM phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol and inositol triphosphate, suggesting that the effect of PIP_2 on gCap39 is specific. PIP_2 dissociated gCap39 from filament ends when added to precapped filaments in the presence of Ca^{2+} (Fig. 3A, curve d_p'). In contrast, gelsolin and villin uncapping by PIP_2 requires a simultaneous decrease in Ca^{2+} concentration (*18*). This difference would allow PIP_2 to uncap gCap39 even when Ca^{2+} concentration is raised, as has been observed during the early phase of stimulation by several agonists. The Ca^{2+}-regulated properties of gCap39 closely resemble that of a 41-kD actin (+) end filament-capping protein isolated previously

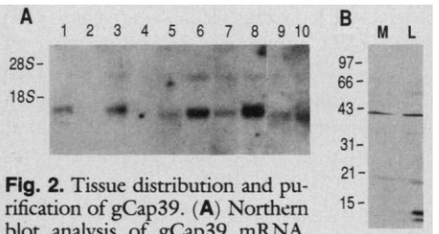

Fig. 2. Tissue distribution and purification of gCap39. (**A**) Northern blot analysis of gCap39 mRNA. Total mouse RNA (15 μg) was loaded in each lane. Lanes 1 to 10, skeletal muscle, liver, stomach, brain, spleen, kidney, heart, lung, intestine, and uterus, respectively. Positions of 28*S* and 16*S* RNA are indicated. (**B**) Purification of gCap39 from RAW 264.7 macrophage lysate (L) and conditioned medium (M). RAW macrophage monolayers grown in Dulbecco's modified Eagle's medium without serum supplement were harvested, and 100,000*g* supernatants were prepared after sonication. Conditioned medium was centrifuged. The lysate (L) and medium (M) fractions were purified by Ca^{2+}-dependent hydrophobic affinity chromatography as described (*13, 14*). The EGTA-eluted fractions were analyzed by SDS–polyacrylamide gel electrophoresis. The prominent low molecular weight doublets are S100. Other proteins eluted are calmodulin (≈20 kD in EGTA), calelectrins (*14*) (67 and 35 kD), and a very small amount of gelsolin [90 kD on SDS-gel (*33*)]. Gelsolin is abundant in macrophages (*4, 34*) but does not bind phenyl-Sepharose efficiently under these conditions. The sizes (in kilodaltons) of standard proteins are indicated. RNA blotted onto GeneScreen Plus (Du Pont Biotechnology Systems) was hybridized with ^{32}P-labeled C15 cDNA in 50% formamide at 42°C.

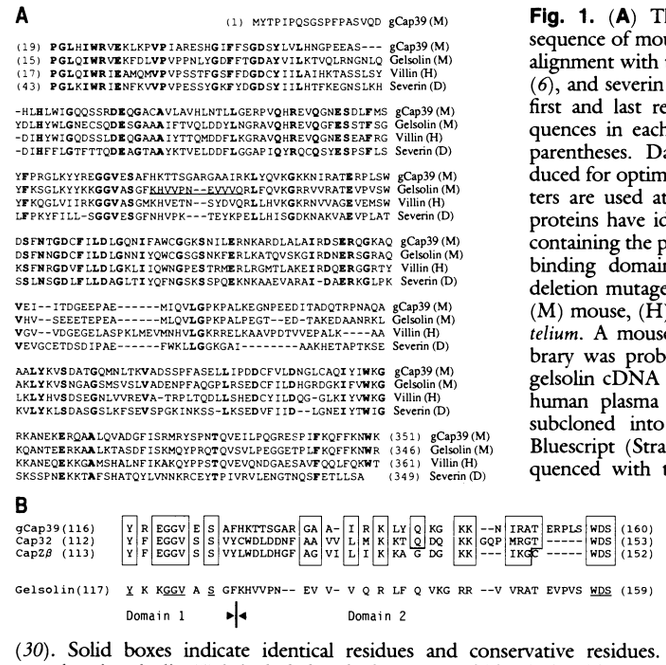

Fig. 1. (**A**) The predicted amino acid sequence of mouse kidney gCap39 and its alignment with those of gelsolin (*9*), villin (*6*), and severin (*10*). The numbers of the first and last residues of the aligned sequences in each protein are indicated in parentheses. Dashes denote gaps introduced for optimal alignment. Bold characters are used at positions where all four proteins have identical residues. Residues containing the putative actin filament side-binding domain of gelsolin defined by deletion mutagenesis (*26*) are underlined. (M) mouse, (H) human, and (D) *Dictyostelium*. A mouse kidney λgt10 cDNA library was probed with a human plasma gelsolin cDNA fragment [bases 1 to 848 human plasma gelsolin (*7*)]. DNA was subcloned into pGEM (Promega) and Bluescript (Stratagene) plasmids, and sequenced with the Sequenase Version 2 system (U.S. Biochemical). (**B**) Similarity between a sequence found in gCap39, the β subunits of *Dictyostelium* Cap32/34 (*29*), and chicken muscle CapZ (*30*). Solid boxes indicate identical residues and conservative residues. The sequence of mouse cytoplasmic gelsolin (*9*) is included at the bottom, and identical residues are underlined. The boundary between domains 1 and 2 (*7*) of gelsolin is indicated.

from rabbit alveolar macrophages by Southwick and DiNubile (*22*) [also called macrophage-capping protein, MCP (*23*)], suggesting that they are related.

gCap39 is unique among the gelsolin family of proteins in that it is primarily a capping protein with no severing activity. Actin filament severing by gelsolin requires cooperative interaction between two actin binding sites (*24*): a PIP_2-regulated filament "side-binding" domain (mapped to between residues 150 and 160 of human plasma gelsolin, underlined in Fig. 1A) (*25, 26*) and a second site (between residues 26 and 139), which causes filament dissociation. Lack of severing may be due to alterations in either domain or disruption of their cooperative interactions. It may be significant that the putative side-binding domain in all three severing proteins can only be aligned with gCap39 after introduction of a two– to three–amino acid gap (Fig. 1A).

Besides gCap39 and MCP, most of the nonsevering capping proteins identified thus far are Ca^{2+}-insensitive and heterodimeric, with α and β subunits between 28 and 36 kD in size (*27–31*). gCap39 has little overall similarity to this family of capping proteins, which includes Cap32/34 (*29*) and CapZ (*30*), except for a short sequence (Fig. 1B), which is also present in gelsolin, although to an even lesser extent. This stretch spans the transition between the two actin-binding domains involved in severing by gelsolin, and is likely to be involved in the capping function as well. gCap39 and the Ca^{2+}-insensitive capping proteins have similar affinities for filament ends (*32*) and, given their ubiquitous presence, are likely to coexist in the same cells. Since the latter capping proteins have no apparent regulation, they may serve a structural role stabilizing the actin cytoskeleton, whereas gCap39 has the more specialized function of capping or uncapping filament ends in response to Ca^{2+} and polyphosphoinositide transients.

The activity of gCap39 complements that of gelsolin in a physiologically significant manner. As far as can be determined, gelsolin and villin are uncapped by polyphosphoinositides only when cytosolic Ca^{2+} concentration falls to resting levels. gCap39 uncapping is less restrictive, occurring either when Ca^{2+} falls or when the concentration of PIP_2 rises, both at low and high Ca^{2+}. PIP_2 uncapping of gCap39 may therefore promote actin polymerization during the initial phase of agonist stimulation when intracellular free Ca^{2+} is elevated. The use of actin-binding proteins with overlapping as well as distinct functions and subtle differences in their response to intracellular signals ensures diversity and fine tuning of each step in the actin polymerization cascade.

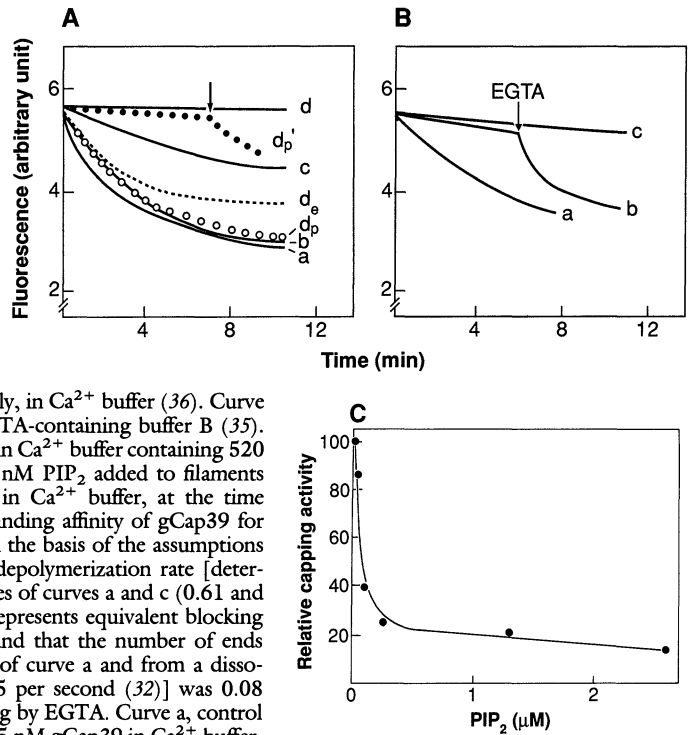

Fig. 3. Functional characterization of gCap39. RAW macrophage medium gCap39 was purified and assayed as described (*35, 36*). Depolymerization of pyrene-labeled actin was monitored as described (*16*). (**A**) Effect of gCap39 on the depolymerization of actin filaments. Curves a, b, c, and d: depolymerization of 1.3×10^{-7} M pyrene-actin in the presence of 0, 1.4, 4.1 and 6.8 nM gCap39, respectively, in Ca^{2+} buffer (*36*). Curve d_e: 6.8 nM gCap39 in EGTA-containing buffer B (*35*). Curve d_p: 6.8 nM gCap39 in Ca^{2+} buffer containing 520 nM PIP_2. Curve d_p': 260 nM PIP_2 added to filaments capped by 6.8 nM gCap in Ca^{2+} buffer, at the time indicated by arrow. The binding affinity of gCap39 for (+) ends was calculated on the basis of the assumptions that the 87% decrease in depolymerization rate [determined from the initial slopes of curves a and c (0.61 and 0.08 nM/s, respectively)] represents equivalent blocking of the (+) filament ends and that the number of ends [calculated from the slope of curve a and from a dissociation rate constant of 7.5 per second (*32*)] was 0.08 nM. (**B**) Reversal of capping by EGTA. Curve a, control actin; curve b, actin with 8.5 nM gCap39 in Ca^{2+} buffer, followed by addition of 1 mM EGTA (arrow); curve c, actin with 8.5 nM gCap39 in Ca^{2+} buffer. (**C**) Inhibition of capping by PIP_2. Increasing amounts of PIP_2 micelles, prepared as described (*18*), were added to 6.8 nM gCap39 in Ca^{2+} buffer, and the rates of actin depolymerization were determined. One hundred percent capping activity was defined as the inhibition of depolymerization observed with 6.8 nM gCap39 in the absence of PIP_2.

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35. RAW macrophage medium gCap39 (shown in Fig. 2B, lane M) was dialyzed against buffer B (2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM adenosine triphosphate, 150 mM KCl, and 20 mM tris-HCl, pH 7.5). Total protein concentration was determined by Bio-Rad assay, and gCap39 concentration was calculated from the relative Coomassie blue staining intensities of the 40-kD band (66%) in the SDS gel of the sample.
36. gCap39 (10 to 50 μl) was placed in a cuvette and the final volume adjusted to 300 μl with buffer B. Calcium chloride was added to a concentration of 1.1 mM (referred to as Ca^{2+} buffer). Pyrene-labeled F-actin (2.5 μl), polymerized with 0.15 M KCl and 2 mM $MgCl_2$, was added to a final concentration of 1.3×10^{-7} M, and the fluorescence was monitored in a Perkin-Elmer LS5 fluorescence spectrophotometer.

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