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Cleavage of La protein by granzyme H induces cytoplasmic translocation and interferes with La-mediated HCV-IRES translational activity

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Granzymes are key components of the cytotoxic arm of the immune response, which play critical roles in eliminating host cells infected by intracellular pathogens and transformed cells. Although the induction of cell death is likely a central process underlying the function of these enzymes, little is known about whether granzymes use additional mechanisms to exert their antipathogen activity. This study identifies La, a phosphoprotein involved in multiple roles in cellular and viral RNA metabolism, as the first nonapoptotic substrate of granzyme H (gzmH), a cytotoxic granule protease that is constitutively expressed by NK cells. Cleavage of La by gzmH occurs at Phe-364 (P_1 site) and generates a COOH-terminal truncated form of La that loses nuclear localization and decreases HCV (hepatitis C virus)-internal ribosome entry site (IRES)-mediated translational activity. The ability of gzmH to cleave host proteins involved in essential viral functions provides a novel mechanism by which granzymes can mediate direct antiviral activities.
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Defense against virally infected and malignant cells depends on the action of cytotoxic cells.[1] Although these use several mechanisms to eliminate target cells, the principal event is the secretion of cytotoxic granules.[2,3] These granules contain the pore-forming protein perforin together with a variety of granule-associated proteases, which include the granzymes (gzms). Perforin provides target cell access and/or trafficking signals for the granzymes, and the granzymes deliver the lethal hit.[4] Humans express five gzms, gzmA and gzmK, which are trypsin-like; gzmB, which cleaves after Asp residues; gzmM, which cleaves after Met and other long unbranched hydrophobic residues, and gzmH, which has chymotrypsin-like activity.[3,5,6] To date, studies have shown that all human gzms contained within cytotoxic granules have the capacity to induce target cell death, and therefore, killing has been considered the central process underlying the antipathogen and antitumor functions of these enzymes.[3]

GzmH is a close structural relative of gzmB that shares high structural homology (71% amino-acid identity), and belong to a tightly linked gene cluster on chromosome 14, which also harbors cathepsin G and mast cell chymase.[7] Although gzmB and cathepsin G are highly conserved between human and mice, the precise functional ortholog of human gzmH has not been determined.[8] Interestingly, gzmH is constitutively expressed in NK cells irrespective of the activation status, and in contrast to GzmB, it is not present in activated $CD8^+$ T cells,[9,10] suggesting that gzmH might play a critical role in the cytotoxic arm of the innate immune response. GzmH has chymotrypsin-like (chymase) activity and is capable of cleaving synthetic substrates with a clear preference of Phe and Tyr in the P_1 site.[5,11] The function of gzmH has just been recently revealed and involves the induction of target cell death, which resembles apoptotic cell death.[10,12] However, the mechanisms and death pathways involved in this process are still controversial.

Once in the cytoplasm of the target cell, gzms activate specific pathways to induce cell death, a potent mechanism to limit viral replication.[2–4] In addition, we have recently shown that gzmH and gzmB can achieve direct antiviral activities through the cleavage of proteins encoded by viruses.[13] In this report, we describe that La, a multifunctional phosphoprotein that plays diverse roles in cellular and viral RNA metabolism, is directly cleaved by gzmH *in vitro* and during cytotoxic-mediated cell death. Cleavage of La by gzmH occurs at Phe-364 (P_1 site), and efficient cleavage absolutely requires a unique extended substrate–enzyme interaction in La,

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Abbreviations: Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; ATP, adenosine triphosphate; BID, BH3 interacting domain death agonist; rCKII, recombinant casein kinase II; DAPI, 4′,6-diamidino-2-phenylindole; DPB, adenovirus DNA-binding protein; E:T, effector:target; FL, firefly luciferase; GFP, green fluorescent protein; GFP-La, GFP-tagged La; gzm, granzyme; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICAD, inhibitor of caspase-activated DNase; IRES, internal ribosome entry site; IVTT, *in vitro* coupled transcription/translation; 100K, adenovirus 100 kDa assembly protein; LAK, lymphokine-activated killer; npLa, nonphosphorylated La; pLa, phosphorylated La; rLa, recombinant La; NK, natural killer; NLS, nuclear localization signal; cNLS, cryptic nuclear localization signal; NoLS, nucleolar localization signal; NRE, nuclear retention element; PCR, polymerase chain reaction; PI, propidium iodide; RL, renilla luciferase; RRM, RNA recognition motif; SDS, sodium dodecyl sulfate; SBM, short basic motif; SV40, simian virus 40; UVB, ultraviolet B; Wt, wild type

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including Thr-362 (P_3 site) and Asp-368 (P_4' site). In addition, phosphorylation of La at Ser-366, a critical change that defines the cellular localization, protein–protein interactions and function[14] protects La against gzmH cleavage. Once cleaved, La is translocated from the nucleus to the cytoplasm, and the truncated product of La has a negative effect on HCV-IRES-mediated translational activity. The direct ability of gzms to cleave host proteins involved in essential viral functions provides a novel antiviral effector mechanism that complements the antipathogen activities of killer cells.

Results

La autoantigen is a target for different death-related proteases. La is a multifunctional protein, which has diverse roles in cellular and viral RNA metabolism, and is a major autoantigen in systemic autoimmune diseases. Like many other autoantigens, La is cleaved by caspases and gzmB.[15–17] Thus, during UVB- and Fas-induced apoptosis (Figure 1a and data not shown, respectively), La is cleaved generating fragments of approximately 46 and 43 kDa (Figure 1a, lane 2). As the caspase inhibitor ac-DEVD-CHO abolished the production of these fragments (Figure 1a, lane 3), these data confirm that the cleavage of La during apoptosis is dependent upon caspase activation. In contrast, during killer cell-mediated cell death, La is cleaved generating the caspase-induced fragments and at least three additional fragments of 40, 31 and 27 kDa (Figure 1b, lanes 3 and 4), which are not induced during UVB- or Fas-mediated death (Figure 1a and data not shown, respectively). As the gzmB inhibitor compound-6[19] abolishes the production of the 31 kDa fragment (Figure 1b, lanes 7 and 8, arrowhead), these data support that it corresponds to the previously described gzmB-induced fragment of La.[15] In addition, the caspase-induced fragments (i.e., 46 and 43 kDa) are importantly decreased as result of gzmB inhibition (Figure 1b, lanes 7 and 8). Interestingly, the generation of the specific cytotoxic lymphocyte-induced fragments of 40 and 27 kDa was not affected through gzmB inhibition (Figure 1b, lanes 7 and 8, open arrows), suggesting that La is cleaved by other components either released or activated by the killer cells.

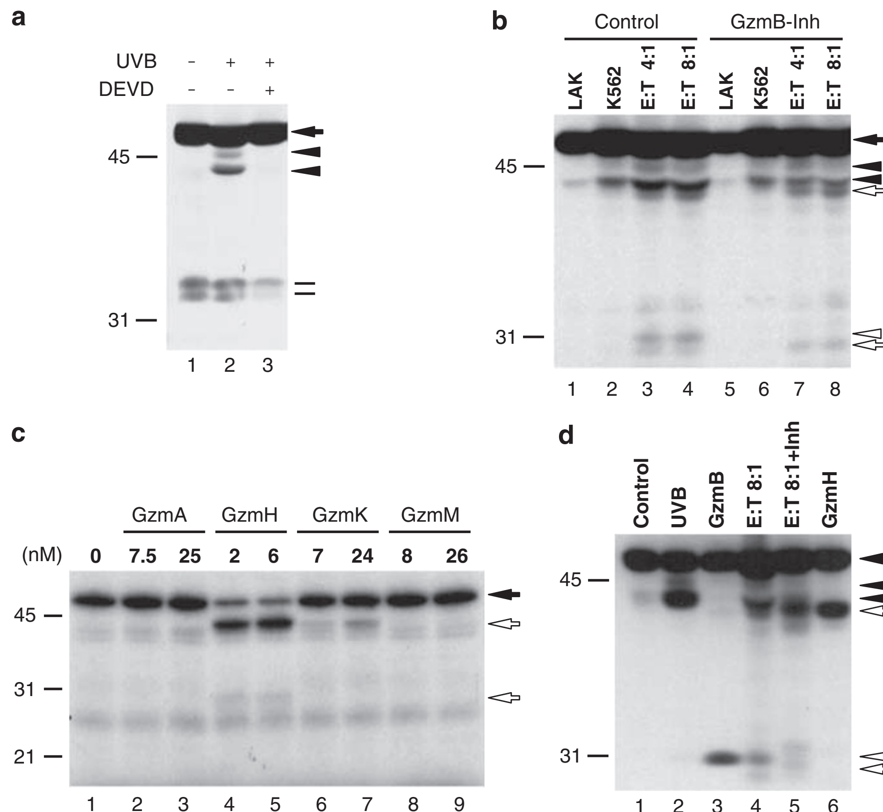


Figure 1 La is cleaved during cytotoxic-mediated cell death. (**a**) Apoptosis was induced by UVB irradiation on HeLa cells as described,[18] in the absence (lane 2) or presence (lane 3) of 100 μM ac-DEVD-CHO. Nonirradiated cells were used as control (lane 1). (**b**) Effector LAK cells (lanes 1 and 5) and K562 target cells (lanes 2 and 6) were incubated alone or coincubated (lanes 3 and 4 and 7 and 8) at increasing E:T ratio for 4 h at 37 °C. In some experiments (lanes 5–8), LAK and K562 cells were incubated with compound 6 (GzmB-inh) before coincubation. (**c**) [^{35}S]methionine-labeled La was incubated in buffer A with increasing amounts of granzymes A, H, K ,and M for 1 h at 37 °C. (**d**) K562 lysates generated in buffer A were incubated in the absence (lane 1) or presence of 200 nM purified gzmB (lane 3) or gzmH (lane 6) for 15 min at 37 °C. In addition, apoptosis was induced in K562 cells by UVB irradiation (lane 2) or by LAK-mediated cytotoxicity (E:T = 8:1) in the absence (lane 4) or presence of compound 6 (E:T = 8:1 + Inh; lane 5). After terminating the reactions, the samples were electrophoresed in adjacent lanes to compare their cleavage patterns. La and its fragments were detected by immunoblotting (**a**, **b,** and **d**) or by fluorography (**c**). The solid arrow denotes intact La, solid arrowheads mark caspase-induced fragments, unfilled arrowhead denotes the gzmB-induced fragment, unfilled arrows mark gzmH cleavage fragments, and the indicator bars denote nonspecific bands detected only in HeLa cells

La is directly cleaved by gzmH. To further address whether component(s) of the granule exocytosis pathway might be responsible for the cleavage of La during cytotoxic-induced cell death, [^{35}S]methionine-labeled La was incubated in the absence or presence of increasing concentrations of purified gzmA, gzmH, gzmK, and gzmM. Interestingly, gzmH directly cleaved La, generating a prominent fragment of 40 kDa and a minor fragment of approximately 27 kDa (Figure 1c, lanes 4 and 5). In contrast, gzmA and gzmM failed to cleave La *in vitro* (Figure 1c, lanes 2–3 and 8–9, respectively), and gzmK slightly cleaved La, but only at high enzyme concentrations (Figure 1c, compare lanes 4 and 7). As La is directly cleaved by gzmH *in vitro* and the fragments generated exactly correspond to those induced in target cells killed by cytotoxic lymphocytes (Figure 1d, compare lanes 4, 5 and 6), taken together, these data strongly support that La is a direct target of gzmH during cytotoxic-mediated cell death.

Definition of the GzmH cleavage site and the extended substrate specificities in La. The specificity of gzmH has been defined using a panel of synthetic peptides.[5,11] The protease has a preference for Phe and Tyr at the P_1 site. On the basis of the cleavage specificity and the size of the most prominent fragment generated by gzmH, potential cleavage sites were predicted. Using site-directed mutagenesis, the candidate P_1 sites were changed, that is, Met-52 to Val, Phe-357 to Gly, and Phe-364 to Val (Met^{52}→Val, Phe^{357}→Gly, and Phe^{364}→Val, respectively), and susceptibility of wild type and mutated forms to cleavage by gzmH was assessed. In the presence of gzmH, cleavage of the mutant Phe^{364}→Val, but not Met^{52}→Val or Phe^{357}→Gly was importantly abolished (Figure 2a), defining Phe-364 as the major gzmH cleavage site in La. As the generation of the minor 27 kD fragment of La is not affected by this mutation, it suggests that gzmH cleaves La at least in two different sites.

Understanding the substrate specificity of gzmH might help to identify natural targets as well as develop specific inhibitors. The extended P_4-P_3-P_2-P_1 substrate specificities of all human gzms have been defined using combinatorial libraries of protease substrates.[11] In La, the gzmH P_4-P_3-P_2-P_1 substrate specificities are Lys-Thr-Lys-Phe, respectively (Figure 2b). Interestingly, Lys at P_4 and P_2 are not well tolerated in the combinatorial tetrapeptide library.[11] To better understand the

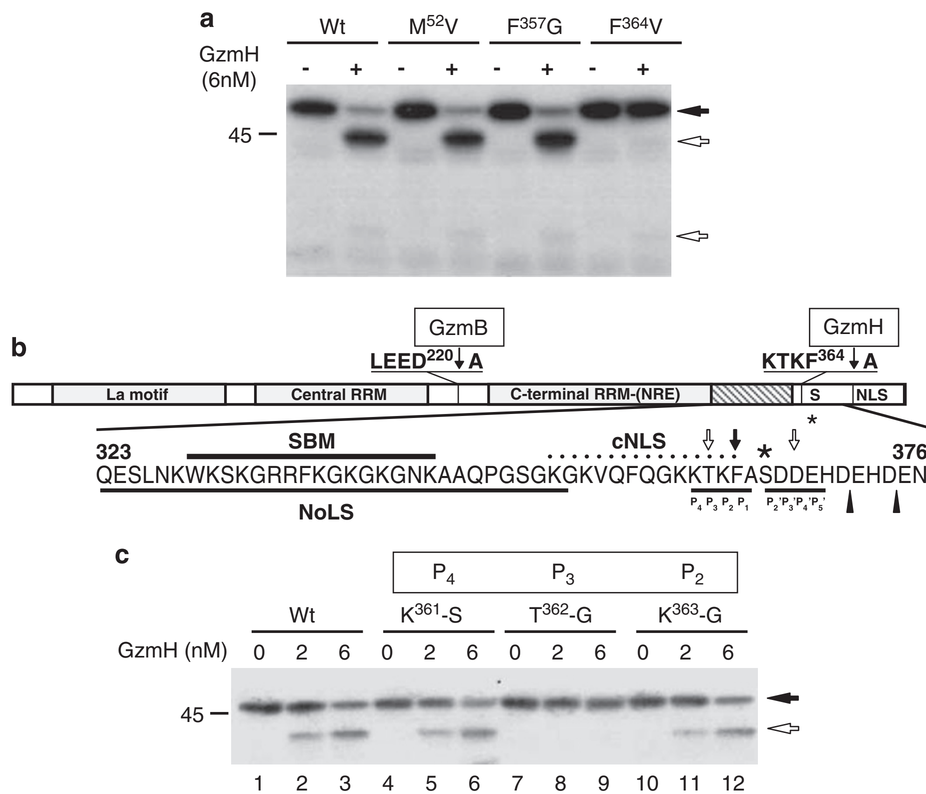


Figure 2 GzmH cleavage site and the extended substrate specificities in La. (**a**) The gzmH cleavage site in La was defined by mutating the P_1 residues Met^{52}→Val, Phe^{357}→Gly, and Phe^{364}→Val, and incubating wild type (wt) and mutated [^{35}S]methionine-labeled products in buffer A with or without gzmH for 60 min at 37 °C. (**b**) Schematic representation of the functional domains and the gzmB, gzmH, and caspase cleavage sites in La. The La structure is represented by the La motif and two RRMs (RNA recognition motif). The C-terminal RRM is atypical and associated to a nuclear retention element (NRE, 316–326). The star indicates the phosphorylation site at 366. The trafficking elements of La are also shown, including the nucleolar localization signal (NoLS, 323–354), a C-terminal nuclear localization signal (NLS, 383–408), a cryptic NLS (cNLS, 352–363), and a short basic motif (SBM). The gzmB and gzmH cleavage sites are located at Asp-220 and Phe-364, respectively. The P_4-P_1 and P_2'-P_5' gzmH substrate specificities are underlined. The filled arrow marks the gzmH cleavage site, the unfilled arrows denote the critical P_3 and P_4' gzmH substrate specificities, and the filled arrowheads mark the putative caspase cleavage sites. (**c**) P_4-P_3-P_2 gzmH cleavage specificities in La. The P_4-Lys, P_3-Thr, and P_2-Lys residues were changed to Ser, Gly, and Gly, respectively, and wild-type and mutated [^{35}S]methionine-labeled products were incubated in buffer A with or without gzmH for 60 min at 37 °C. After terminating the reactions, the samples were electrophoresed and La was detected by fluorography. Solid arrow denotes intact La, the unfilled arrow marks the gzmH-induced fragment

gzmH extended substrate specificities in biological macromolecular substrates, the P_4-P_2 positions in La were changed to other nontolerated amino acids according to the residue preferences defined in synthetic substrates.[11] When the P_4-Lys and P_2-Lys were changed to Ser and Gly, respectively, gzmH cleavage of La was unaffected (Figure 2c, lanes 5–6 and lanes 11–12, respectively). In contrast, the change of P_3-Thr to Gly totally abolished the cleavage of La by gzmH (Figure 2c, lanes 8–9). Regarding the gzms substrate preference C-terminal to the scissile bond (prime side), only gzmB has been studied.[20,21] Among the P_1'-P_2'-P_3'-P_4' residues, the presence of acidic P_4' residue likely promotes efficient interaction of gzmB with substrates through the formation of a salt bridge with Lys-27 in gzmB. Interestingly, Lys-27 is shared among the gzmH and gzmB sequences, and the P_3', P_4', P_5' residues at the gzmH cleavage site of La correspond to the acidic residues Asp, Asp and Glu, respectively (Figure 2b). When Asp-367 (P_3'), Asp-368 (P_4') or Glu-369 (P_5') were changed to alanine, only the presence of Ala at the P_4' site decreased the cleavage of La by gzmH (data not shown). However, when double mutants were generated, that is, $Asp^{367}Asp^{368} \rightarrow$ Ala-Ala, $Asp^{368}Glu^{369} \rightarrow$ Ala-Ala and $Asp^{367}Asp^{368}Glu^{369} \rightarrow$ Ala-Asp-Ala, the combination of Ala at P_3' and P_4' or Ala at P_4' and P_5' totally abolished the cleavage of La by gzmH (Figure 3a, lanes 5–6 and lanes 8–9, respectively). In contrast, when Asp-368 was flanked by Ala mutations at P_3' and P_5', cleavage of La by gzmH was only minimally affected (Figure 3a, lanes 11–12). In summary, gzmH directly cleaves La at Phe-364, and extended substrate specificities require Thr at the P_3 site, and Asp at $P_{4,}'$ which can be partially replaced when acidic residues are present at both the P_3' and P_5' sites.

Phosphorylation of La on serine 366 and cleavage susceptibility by GzmH. Although most La is phosphorylated on Ser-366 and resides in the nucleoplasm, nonphosphorylated La (npLa) is concentrated in nucleolar sites where it is likely involved in ribosome biogenesis.[22] Interestingly, Ser-366 corresponds to the P_2' residue at the gzmH cleavage site in La (Figure 2b). To determine the consequences of Ser-366 phophorylation in the cleavage of La by gzmH, rLa was phosphorylated *in vitro* with casein kinase II (CKII),[14] and the gzmH cleavage efficiency of phosphorylated La (pLa) and nonphosphorylated La (npLa) were compared. In the presence of increasing concentrations of gzmH, npLa was very efficiently cleaved (Figure 3b, lanes 2–5). In contrast, pLa was resistant to cleavage by gzmH (Figure 3b, lanes 7–10). When Ser-366 was changed to Glu, the cleavage efficiency of La by gzmH was similar to the wild type, suggesting that the gzmH resistance induced by phosphorylation at Ser-366 is the result of steric hindrance, but not charge (Figure 3c).

The N-terminal domain generated by gzmH cleavage of La loses its nuclear localization activity. La contains a multitude of closely associated and sometimes overlapping localization signals in the C-terminal domain (Figure 2b)—a nuclear retention element, a nucleolar localization signal, a cryptic NLS, and a nuclear localization signal.[22] Interestingly, it appears that the localization activities of these signals involve complex functions that remain to be fully understood. Thus, although truncated GFP-La containing amino acids 1–363 has mainly nuclear localization (as it still contains the NRE, NoLS, and cNLS),[22] two different proteolytic cleavages of La induced by poliovirus protease 3C[23] and caspase-3[16,17] (positions 358 and 374, respectively) showed cytoplasmic accumulation, despite containing both the NRE and the NoLS, and for the caspase fragment also the cNLS. To address the effect of gzmH cleavage of La on its cellular localization, we generated truncated GFP-La, containing amino acids 1–364 (GFP-La 1–364). Interestingly, when GFP-La 1–364 was expressed in HeLa cells, its cellular localization was cytoplasmic, whereas GFP-La wild type

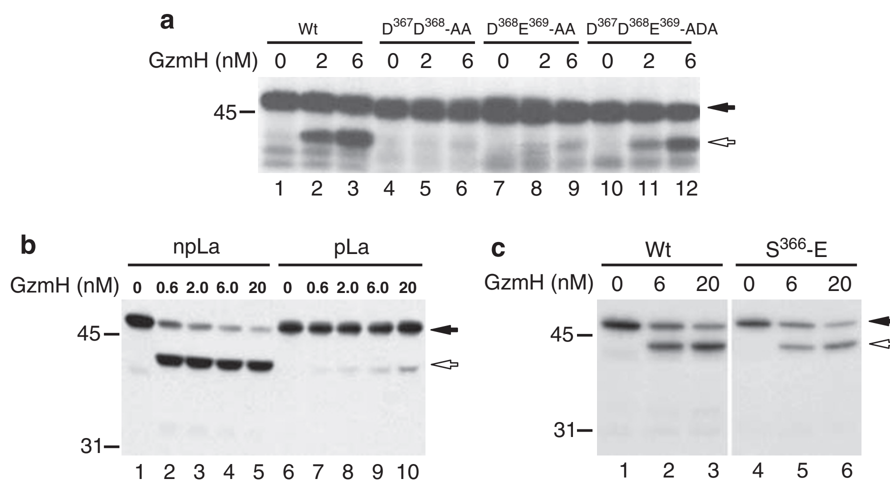


Figure 3 Prime side preferences of gzmH in La. (**a**) The P_3'-P_4' (lanes 4–6), P_4'-P_5' (lanes 7–9), and $P_{3'}$and $P_{5'}$ (lanes 10–12) residues were mutated ($Asp^{367}Asp^{368} \rightarrow$ Ala-Ala, $Asp^{368}Glu^{369} \rightarrow$ Ala-Ala and $Asp^{367}Asp^{368}Glu^{369} \rightarrow$ Ala-Asp-Ala, respectively), and wild-type and mutated [^{35}S]methionine-labeled products were incubated in buffer A in the absence or presence of gzmH for 60 min at 37 °C (**b**) npLa (lanes 1–5) and pLa (lanes 6–10) were incubated in the absence or presence of increasing amounts of purified gzmH. (**c**) The P_2' (Ser-366) residue in La was mutated to Glu. Then, the wild-type (lanes 1–3) and mutated (lanes 4–6) [^{35}S]methionine-labeled products were incubated in buffer A with or without gzmH for 60 min at 37 °C. After terminating the reactions, the samples were electrophoresed and La was detected by immunoblotting (**b**) or by fluorography (**a** and **c**). Solid arrow denotes intact La and the unfilled arrow marks the gzmH-induced fragment

was concentrated in the nucleus (Figure 4, compare panels d and h). As it has been previously described that GFP-La, containing amino acids 1–363, has nuclear localization,[22] we also generated GFP-La 1–363. Interestingly, in contrast to an earlier report,[22] GFP-La 1–363 also showed cytoplasmic localization (data not shown).

To further address the consequences of cytotoxic granule-mediated killing in the cellular localization of La, K562 target cells were killed by LAK cells, and endogenous La in the target cells was analyzed by immunofluorescence. Thus, in contrast to control cells in which La has nuclear localization (Figure 5j), in target cells, killer lymphocytes induce nuclear condensation and fragmentation (Figure 5e, k, m and n), and La is translocated into the cytoplasm (Figures 5h, k, m and n). As La is cleaved by caspases, gzmB and gzmH, we used the potent gzmB inhibitor compound-6 (which also shares inhibitory activity against caspases 3 and 8)[19] to dissect the caspase- and gzmB-independent processing of La during cytotoxic lymphocyte-induced cell death. In contrast to killer cells with full gzms activity, in the presence of compound-6, killer cells induce perinuclear chromatin condensation in target cells (Figure 5, compare panels e and f). In addition, La showed both nuclear and cytoplasmic redistribution in target cells (Figure 5i, l, o and p), likely because gzmH only

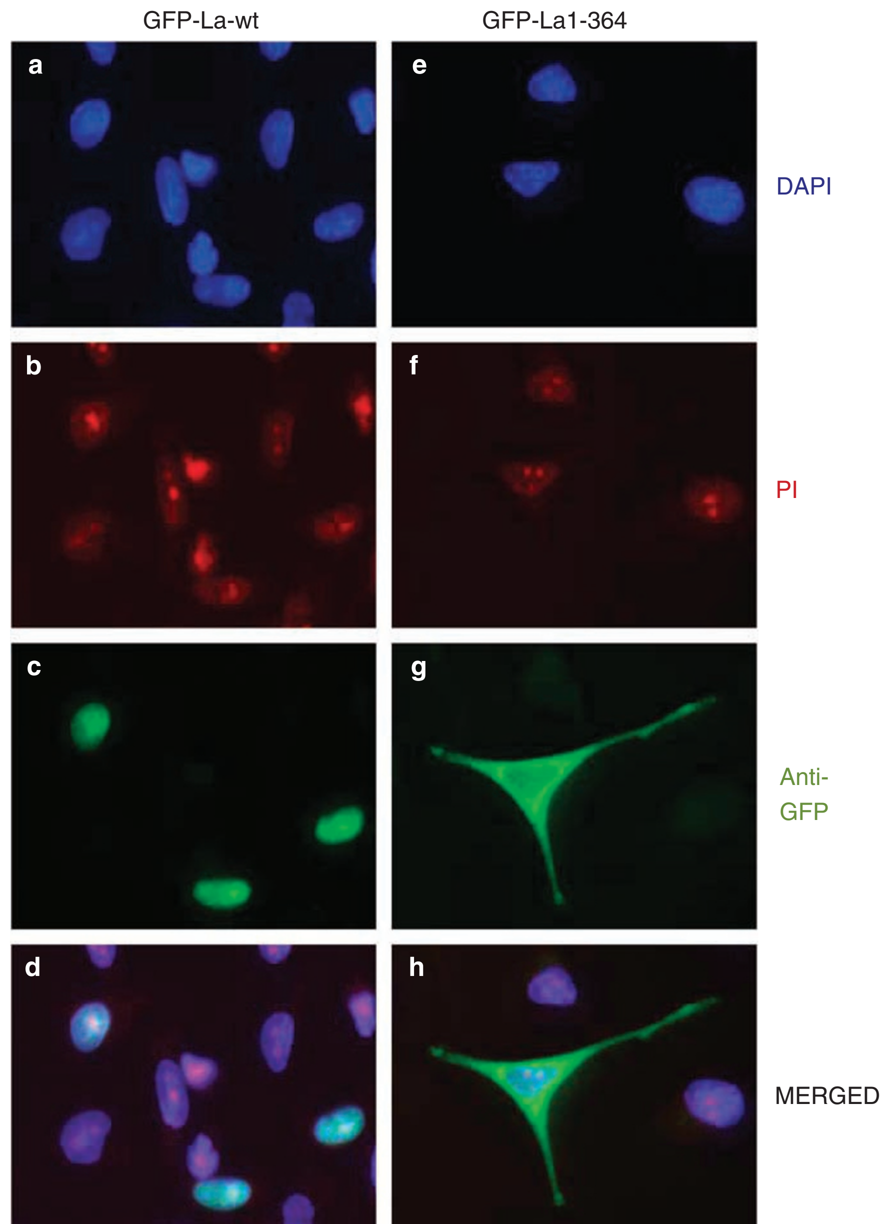


Figure 4 The N-terminal domain generated by gzmH cleavage of La loses its nuclear localization activity. HeLa cells were transiently transfected to express GFP-La-wt (**a**–**d**) or GFP-La 1–364 (**e**–**h**). After 48 h, the cells were methanol fixed and permeabilized, and stained with a rabbit polyclonal antibody against GFP (Invitrogen) and Alexa Fluor 488 donkey antirabbit antibodies (Invitrogen), as well as propidium iodide (PI) and DAPI. Individual (**a**–**c** and **e**–**g**) and merged images (**d** and **h**) of DAPI (blue), PI (red), and GFP (green) staining are shown

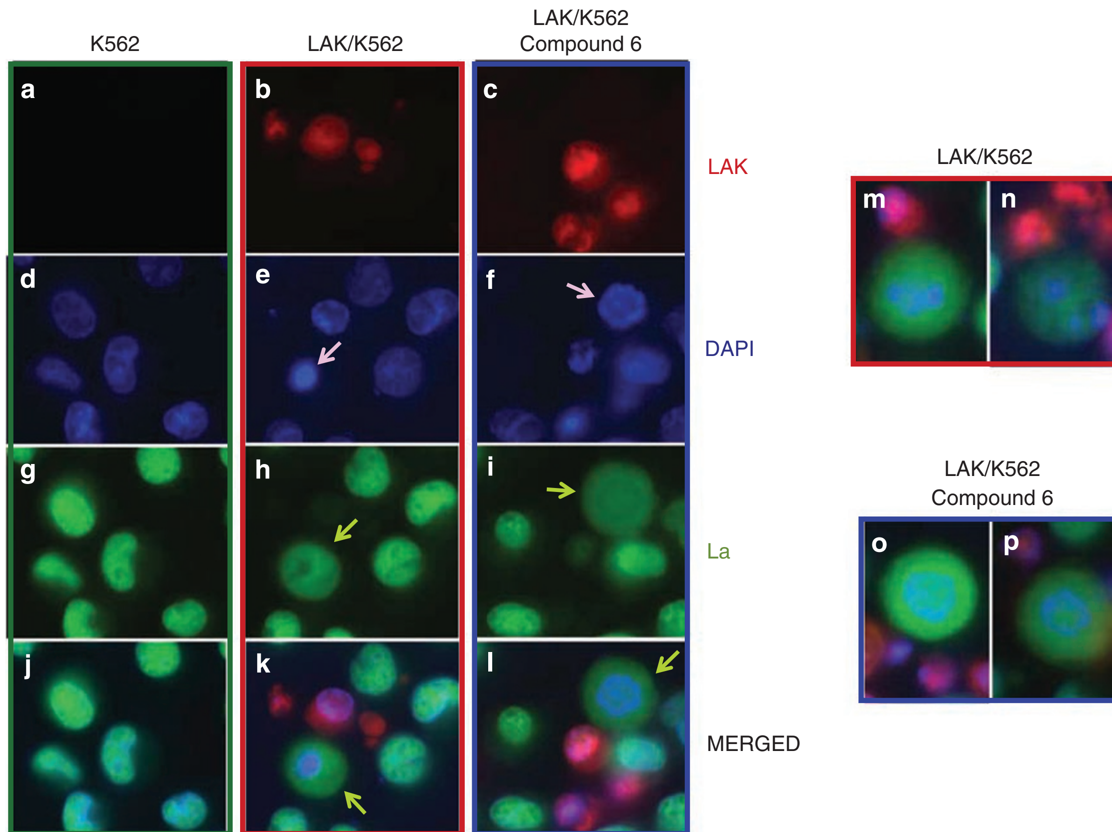
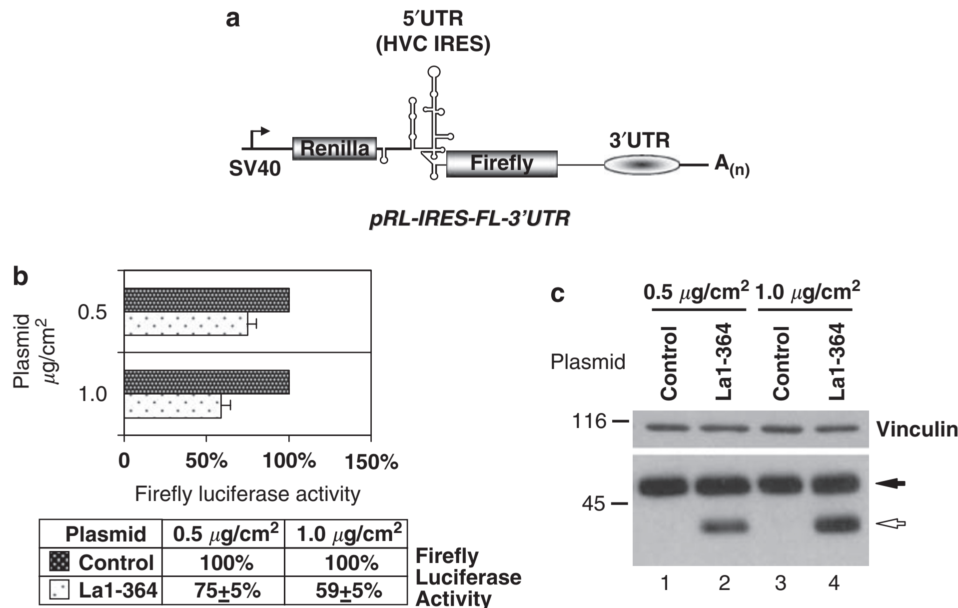


Figure 5 La is translocated from the nucleus to the cytoplasm in target cells killed by LAK cells. LAK cells were prelabeled with SNARF-1 carboxylic acid (Invitrogen) as described by the manufacturer, and further coincubated with K562 target cells at E:T ratio of 4:1 (red outlined panel). In some experiments, prelabeled LAK and K562 cells were incubated with compound 6 (GzmB-inhibitor) before coincubation (blue outlined panel). As controls, K562 cells were incubated alone (green outlined panel). After 3 h, the cells were bound to Histogrip (Invitrogen)-coated microscope slides, then methanol fixed and permeabilized, and stained with the monoclonal antibody, SW5,[24] against La and Alexa Fluor 488 goat antimouse IgG2b antibodies (Invitrogen), as well as DAPI. Individual (**a**–**i**) and merged images (**j**–**p**) of LAK (red), La (green), and DAPI (blue) staining are shown. The arrows in panels e and f mark nuclear changes in LAK-killed target cells in the absence or presence of gzmB inhibition, respectively, and the arrows in panels h, i, k and l denote cells in which La has been translocated from the nucleus to the cytoplasm in target cells killed by LAK cells. Panels m–p show merged images of representative LAK-killed target cells in the absence (**m** and **n**) and presence (**o** and **p**) of gzmB inhibition

targets a fraction of La (i.e., npLa). In summary, these experiments showed that cytotoxic lymphocytes induce the nuclear loss of La in target cells under conditions of caspase- and gzmB-independent killing. As gzmH is the only additional protease capable of directly and efficiently processing La into a fragment that shows cytoplasmic redistribution (Figure 4, panel h) during cytotoxic-mediated killing, La is most likely a genuine target and a physiological substrate for gzmH.

Truncated La 1–364 interferes with HCV-IRES translational activity. La is an RNA-binding phosphoprotein that functions in many aspects of RNA metabolism. It is a putative transcription factor for RNA polymerase III, an RNA processing regulator, a possible export and nuclear retention factor, and a regulator of viral RNAs and ribosomal protein transcripts in the cytoplasm.[25] Moreover, La stimulates IRES-mediated translation of virally encoded genes in cells infected by viruses including HCV, poliovirus, coxsackievirus B3 and HIV.[26–29] As cytotoxic cells are key components of the immune response that play important roles in eliminating intracellular pathogens, we wondered whether cleavage of La by gzmH might affect the viral IRES-mediated translational activity of La. To directly address the functional consequences of La processing by gzmH on viral IRES-mediated translation, we used a bicistronic HCV-IRES reporter construct (i.e., pRL-IRES-FL-3′UTR), whose SV40 promoter drives cap-dependent translation (i.e., La independent) of renilla luciferase and cap-independent HCV-IRES-mediated translation (i.e., La dependent) of firefly luciferase (Figure 6a).[30] 293T cells were transiently co-transfected with pRL-IRES-FL-3′UTR and either with a control plasmid (pcDNA3.1) or with a plasmid-expressing La 1–364 (Figure 6b). The expression level of endogenous La and La 1–364 were monitored by western blot (Figure 6c), whereas renilla and firefly luciferase expressions were quantified by the activity measurements. In contrast to other fragments generated from caspase or gzm-induced cleavage of death substrates,[3,31–35] the gzmH-induced fragment of La had no evident effect on the morphology or cell survival when expressed in cells by transfection (Figure 4h and data not shown). However, we noted that the expression of increasing amounts of La 1–364 decreased the expression of IRES-dependent translation of firefly luciferase (Figure 6b). Hence, this finding strongly supports the idea that the gzmH-specific cleavage of La exerts a dominant-negative effect against HCV-IRES-directed activity.



Plasmid	0.5 μg/cm²	1.0 μg/cm²	
Control	100%	100%	Firefly Luciferase Activity
La1-364	75±5%	59±5%	

Figure 6 La 1–364 interferes with HCV-IRES-mediated translational activity. (**a**) Schematic representation of the bicistronic HCV-IRES reporter construct driven by the SV40 promoter to direct cap-dependent translation of renilla luciferase and cap-independent HCV-IRES-mediated translation of firefly luciferase. (**b** and **c**) 293T cells were cotransfected with 0.1 μg/cm^2 of pRL-IRES-FL-3′UTR vector and 0.5 or 1.0 μg/cm^2 of control (pcDNA 3.1) or La 1–364 plasmids. At 48 h post-transfection, cells were lysed and the luciferase activities were determined (**b**), as well as the expression of endogenous and 1–364 La (**c**). In panel b, the mean firefly luciferase activity from control (i.e., pcDNA3.1)-transfected cells was set at 100% and used to normalize the firefly luciferase activities of La 1–364 cells. The resulting luciferase values are shown as percentages relative to control-transfected cells (mean ± S.D.). In panel c, endogenous and 1–364 La were detected by immunoblotting using a human monospecific polyclonal antiserum. Vinculin detected by immunoblotting is shown as sample loading control. The solid arrow denotes endogenous La, the unfilled arrow marks La 1–364

To further address the consequences of cytotoxic granule-mediated killing against HCV-IRES-mediated translation, pRL-IRES-FL-3′UTR-expressing target cells were killed by LAK cells in the absence or presence of compound-6, and renilla and firefly luciferase were quantified. Interestingly, both cap-dependent and IRES-dependent-mediated translations were dramatically suppressed in target cells killed by LAK cells, both in the absence and presence of compound-6 (data not shown). As LAK cells contain all gzms, it is not surprising that killer cells may execute potent antitranslational activities (both cap-dependent and IRES-dependent) against target cells, in a process that is independent of gzmB activity. These data, in concert with our earlier work,[13,36] strongly support the idea that the combinatorial effect of multiple gzms completely paralyzes the pathways in target cells and overcomes the effect of gzm inhibitors. Understanding the components of such paralysis remains very important.

Discussion

Granzymes are proteases released from cytotoxic-lymphocyte granules into target cells to protect mammals from virus infection and transformed cells. Although the induction of target cell death has been considered as the central mechanism by which gzms accomplish their antiviral activity, recent evidence suggests that gzm-mediated cleavage and inactivation of viral proteins is a death-independent mechanisms, which can directly and efficiently affect the viral life cycle.[13] The study reported here identifies La as the first nonapoptotic substrate of gzmH and provides evidence in support to the idea that, apart from their apoptosis inducing potential, gzms possess additional antiviral activities by intercepting critical cellular steps of viral replication.

Granzyme H belongs to a family of five human serine proteases that are expressed by cytotoxic cells, and it has been the last gzm (in humans) in which its citotoxic function has been revealed. Two independent studies have recently shown that recombinant purified gzmH is able to induce cell death when delivered *in vitro* into target cells, and the death process appears similar to apoptosis. However, both reports are quite different regarding the mechanisms that gzmH uses to execute death. While Fellows *et al.*[10] observed slow target cell death with gzmH that was independent of caspase, BID and ICAD cleavage, Hou *et al.*[12] observed a rapid type of cell death that depended on a direct cleavage of caspase-3, BID and ICAD by gzmH. Besides the major differences in the production of the recombinant enzymes, the artificial systems used for intracellular protease delivery and the use of likely nonphysiological concentrations of gzmH, there is not a clear explanation of why the enzymes of each research group might have different biological activities *in vitro*. In this report, we have used primary effector killer cells to deliver physiologic concentrations of granule proteases into target cells and simulate *in vivo* cytotoxic cell-mediated killing. Using a dual biochemical and cellular approach and purified components to evaluate potential gzmH–substrate interactions *in vitro*, we arrived at the conclusion that La is a direct and biological relevant target for gzmH.

Besides the uncertain cleavage of caspase-3, BID and ICAD,[10,12,13] no cellular substrates for gzmH have yet been identified. La was first described as a human autoantigen more than 20 years ago, and has been identified in many

eukaryotes from yeasts to humans. The C-terminal region is the least conserved part of the La protein, varying in both size and sequence between species.[25] This portion of the La protein usually contains between 40 and 50% charged residues. In addition, it contains a multitude of closely associated and sometimes overlapping functional and post-translational regulatory regions (Figure 2b), including nuclear and nucleolar localization signals and multiple phosphorylation sites (i.e., Thr-302, Ser-325 Thr-362, and Ser-366).[22,25,37] Interestingly, gzmH cleaves La at the C-terminal region, and its cleavage efficiency depends on unique features found in this domain. Thus, the presence of a track of negatively charged residues C-terminal to the scissile bond (from P_3' to P_5') ensures efficient cleavage, likely by promoting efficient interaction of gzmH with La through the formation of a salt bridge with Lys-27 in gzmH. In addition, the cleavage site in La is delimited by two residues, which are targets for phosphorylation, Thr-362 (P_3 site) and Ser-366 (P_2' site). In this regard, the presence of Thr at the P_3 site is absolutely required for gzmH cleavage of La, and it is, therefore, likely that phosphorylation at this site is not compatible with its cleavage specificity. Similarly, phosphorylation at Ser-366, a critical change that defines the cellular localization, protein–protein interactions and function of La,[14] directly blocks the cleavage of La by gzmH likely through steric hindrance. Thus, it is probable that the initial cleavage of La by gzmH might be limited to certain molecular variants that are not modified by phosphorylation. However, as La is dephosphorylated during apoptosis,[17] cleavage of La by gzmH is likely to be amplified as the cell death program progresses. Once La is cleaved, it loses its major NLS, which promotes the translocation of La from the nucleus into the cytoplasm. Alternatively, La relocation may coincide with gzmH-induced cell death.

During the induction of protease-mediated death (e.g., caspases or gzms), cleavage and inactivation of essential host proteins involved in cell cycle, cell repair, and protein synthesis (among others) are the hallmarks to ensure the killing efficiency induced by death proteases.[6] Thus, following the 'death protease rule', the direct cleavage of La induced by gzmH during cytotoxic-mediated cell death is likely to be only one part of the broad amplifying death process induced by this protease. However, it is noteworthy that by contrast to other death substrates, La can be cleaved by three different death proteases, including effector caspases,[16,17] gzmB,[15] and gzmH, suggesting that this molecule is a critical mechanistically relevant target during cell death and other antiviral responses. To this end, we explored the effect of La cleavage on specific cellular functions, which are not directly linked to the process of cell death, but might be crucial in the antipathogen activity of cytotoxic cells. Interestingly, although the N-terminal fragment of La generated by gzmH cleavage has no evident effect in cellular metabolism when expressed by transfection, it has a dominant-negative activity against HCV-IRES-mediated translation. Thus, these data strongly suggest that in addition to the induction of target cell death, the direct cleavage of cellular substrates by gzms might represent a rapid and efficient mechanism to block or slow down the spreading of intracellular pathogens, independently of the time or capacity of the target cell to die. In this regard, we have recently showed that during cytotoxic-mediated cell killing of adenovirus type 5-infected target cells, gzmH directly cleaves the adenovirus DNA-binding protein (DBP; a viral component absolutely required for viral DNA replication), and thereby induces a rapid decay of viral DNA in the infected target cell.[13] In addition, gzmH also cleaves the adenovirus-encoded 100K inhibitor of gzmB,[38] allowing the recovery of gzmB activity, which further targets the same viral products (i.e., DBP and 100K), amplifying the antiviral process initiated by gzmH.[13] As viruses have evolved multiple mechanisms to block intracellular death pathways activated by gzms,[6,13,39] the direct gzm-mediated cleavage and inactivation of viral and host proteins involved in essential viral functions may represent an important additional defense tool to suppress the intercellular dissemination of intracellular pathogens.[36]

Materials and Methods

Human recombinant gzms. Granzymes H, K, and M were expressed as inclusion proteins, refolded and converted into the active mature form as described.[10] The gzmB was a gift from Nancy Thornberry (Merck Research Laboratories., Rahway, NJ, USA). The gzmA was purchased from Alexis Biochemicals.

Cell culture and cell transfection. HeLa, 293T and K562 cells were cultured using standard procedures. Cell lines were transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

Analysis of cytotoxic lymphocyte-induced cell death. Lymphokine-activated killer cells were prepared as described.[40] Effector LAK cells and K562 target cells were incubated alone or coincubated for 4 h at 37°C. The E:T ratios are specified in the figure legends. When required, effector and target cells were preincubated for 1 h in the absence or presence of 20 μM compound 6 followed by the coincubation of the effector/target cell . After washing, cells were immediately lysed in SDS sample buffer and boiled. Samples were analyzed by electrophoresis on 8.5% SDS-polyacrylamide gels. La was visualized by immunoblotting as described,[40] using a human monospecific polyclonal antiserum.

Purification of rLa protein, phosphorylation with CKII, and cleavage by gzmH. cDNAs encoding wild-type La followed by a His-6 tag was generously provided by Richard Maraia (National Institutes of Health, Bethesda, MD, USA). Purification was achieved by nickel affinity resin according to the manufacturer's instructions (Qiagen). *In vitro* kinase assays were performed as described[14] with slight modifications. In all, 10 μl reactions containing a premixed pool of 500 ng of rLa, 0.2 mM ATP, 10 mM $MgCl_2$ and 10 mM Tris-Cl (pH 7.5) were incubated in the absence (npLa) or presence (pLa) of 4 U of rCKII (New England Biolab). After 45 min at 30 °C, 50 ng of npLa or pLa were incubated in buffer A (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40) containing 0.05% ovalbumin (as carrier) in the absence or presence of increasing amounts of purified gzmH. After 15 min, at 37 °C, reactions were stopped by adding SDS-loading buffer and boiling. Samples were analyzed by electrophoresis on 10% SDS-polyacrylamide gels and La, and its fragments were visualized by immunoblotting using a human monospecific polyclonal antiserum.

Cleavage of *in vitro* generated La. La cDNA was generously provided by Daniel Kenan, cloned into pcDNA3.1 (Invitrogen), and used as template for *in vitro* coupled transcription/translation (IVTT; Promega) to generate [^{35}S]methionine-labeled La. Cleavage reactions were performed in buffer A. Amount of proteases and length of incubation are denoted in figure legends. After terminating the reactions by adding SDS sample buffer and boiling, samples were electrophoresed on 10% SDS-polyacrylamide gels. Radiolabeled proteins and their fragments were visualized by fluorography.

Determination of the gzmH cleavage site, the gzmH extended substrate specificities in La, and generation of GFP-La 1–364 and GFP-La 1–363. La cDNA cloned into pcDNA3.1 was used as template for site-directed mutagenesis by overlap extension PCR (Strategene). [^{35}S]methionine-

labeled La was generated by IVTT and used as substrate for cleavage assays containing gzmH and analyzed as described above. To generate GFP-La 1–364 and GFP-La 1–363, La cDNA cloned into pEGFPC1 was used as template for site-directed mutagenesis in which the sequence that encodes for Ala at position 365 or Phe at position 364 were changed for Amber or Ochre termination signals, respectively. cDNAs sequences were confirmed after mutagenesis.

Reporter assay. Using Lipofectamine 2000 (Invitrogen), 293T cells were transiently cotransfected with 0.1 μg/cm^2 of pRL-IRES-FL-3′UTR vector and 0.5 or 1.0 μg/cm^2 of control (pcDNA 3.1) or La 1–364-expressing plasmids. At 48 h post-transfection, cells were harvested and used for immunoblot analysis, as well as to determine renilla and firefly luciferase activities using the Dual-GLO Luciferase Assay System (Promega). Both luciferase activities were normalized to control (nontransfected cells) values. Then, changes in HCV-IRES-directed firefly luciferase activities relative to cap-dependent renilla luciferase (i.e., firefly luciferase/renilla luciferase) activities were determined. The mean firefly luciferase activity from control (pcDNA 3.1)-transfected cells was set at 100% and used to normalize the firefly luciferase activities of La 1–364 expressing cells.

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