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A Switching System Regulating Subcellular Localization of Nuclear Proteins Using a Viral Protease

Masashi Urabe,*,[1] Akihiro Kume,* Tohru Takahashi,† Nobufusa Serizawa,† Kiyotake Tobita,‡ and Keiya Ozawa*,§

**Division of Genetic Therapeutics, Center for Molecular Medicine, ‡Department of Virology and §Department of Hematology, Jichi Medical School, Tochigi, Japan; and †Biomedical Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan*

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We explored a novel approach to the functional regulation of nuclear proteins; altering their subcellular localization. To anchor a nuclear protein, β-galactosidase with the nuclear localization signal of SV40 (nβ-gal), within the cytoplasm, nβ-gal was fused to the transmembrane domain of granulocyte colony-stimulating factor receptor (G-CSFR), a membrane protein. To liberate the nβ-gal portion from the fusion protein, we used a protease derived from a plant virus, whose recognition sequence was inserted between the G-CSFR and nβ-gal. Western analysis showed that the chimeric protein was cleaved in the presence of the protease in 293 cells and that the fusion protein without the recognition sequence remained intact. This chimeric protein was localized exclusively in the cytoplasm as visualized by X-gal staining and immunofluorescence microscopy. In contrast, when expressed together with the protease, β-gal was predominantly detected in the nuclei. Moreover, we isolated 293-cell clones constitutively expressing the protease, indicating that this protease is not cytotoxic. These results suggest that the viral protease-mediated alteration of subcellular localization can potentially regulate the function of nuclear proteins.

Key Words: **nuclear protein; subcellular localization; viral protease.**

Recent advances in recombinant DNA technologies have made it possible to regulate the function of proteins through various kinds of genetic manipulation. It is believed that a cellular protein is transported from its site of synthesis to a site where it can function appropriately. The transportation of a newly synthesized protein is mediated by a specific motif of amino acid residues within the protein, such as leader sequences targeting to secretion vesicles, membranes or mitochondria, and nuclear localization signal (1, 2). This would suggest that modification of the transportation signal can cause a protein to be transported to some other site, a process by which the function of the protein could be regulated.

Since most of the nuclear proteins play key roles in cellular function such as proliferation, differentiation, maturation, and death, controlling their functions would not only elucidate unknown phenomena associated with them, but also develop a new strategy. This method would be particularly valuable for cytotoxic or proapoptotic nuclear proteins, because it is impossible otherwise to maintain the cells expressing such proteins.

One of the widely used regulatory systems for the protein of interest is fusing the protein to the ligand-binding domain of the steroid hormone receptor (3). The chimeric proteins have been shown to function in a hormone-dependent manner and this strategy has contributed to analyzing the function of a number of nuclear proteins, such as oncogene products (4).

In the present study, we explored a novel approach to the functional regulation of nuclear proteins through altering their subcellular localization. To illustrate this concept, a model experiment was conducted. To trap a nuclear protein, β-galactosidase with the nuclear localization signal of SV40 T antigen (nβ-gal), within the cytoplasm, nβ-gal was fused to the transmembrane domain of a membrane protein, granulocyte colony-stimulating factor receptor (G-CSFR). To liberate the nβ-gal portion from the chimeric protein, we used a protease derived from a plant virus, clover yellow vein virus, whose recognition sequence was inserted between G-CSFR and nβ-gal. We postulated that once nβ-gal anchored within the cytoplasm is released by

[1] To whom correspondence should be addressed: Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minami-kawachi, Tochigi 329-0498 Japan. Fax: 81-285-44-8675. E-mail: murabe@jichi.ac.jp.

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the protease, it would migrate into the nuclei and function normally.

Clover yellow vein virus (ClYVV), a member of the genus *Potyvirus*, infects plants (5). The genome is a single-stranded RNA of approximately 10 kb with positive polarity, which codes for a large polyprotein of 3072 amino acids. This large protein with a single open reading frame is proteolytically cleaved into mature functional polypeptides by proteases encoded by the virus. One of the viral proteases, nuclear inclusion-a (NIa) protease, recognizes a consensus sequence, F(M)Q(E)/S(G,A), where the amino acids in parenthesis represent alternatives, and cleaves at that site by *cis*- and *trans*-acting proteolysis. A homology search with other potyviruses shows that the mature ClYVV-NIa protease consists of 435 amino acids. A deletion analysis of NIa protease of tobacco etch virus (TEV), another potyvirus, demonstrated that a bipartite nuclear localization signal exists in the N-terminal portion (6). On the other hand, a site-directed mutagenesis study showed that the catalytic domain is in the N-terminal half (7). A possible cleavage site is also identified within the NIa polypeptide, where the NIa protease cleaves itself into the N-terminal portion and C-terminal catalytic domain (8). The ClYVV-NIa also has a possible cleavage site within the molecule. The proteolytic product derived from the C-terminal half including the catalytic domain consists of 243 amino acid residues (27 kDa).

The NIa protease derived from TEV was applied to the removal of affinity tags from recombinant proteins (9). In addition, a chimeric protein with the NIa protease and interleukin-11 produced in *Escherichia coli*, has been shown to produce mature interleukin-11 by autoproteolysis (10).

MATERIALS AND METHODS

Plasmid construction. Primers used in this study were: A1, 5′-ATATGCGGCCGCATGTCCTTTACTTTGACCAAC; A2, 5′-GCTCATCGATAATTTCACCGC; A3, 5′-ATGCTTCGAAGCATGCGCGGCCCTCCAAAAAAGAAG; B1, 5′-GGCCCCAGCCAGATGAAGTTTGTGTTCCAGAGCGACGGCATGCT; B2, 5′-CTAGAGCATGCCGTCGCTCTGGAACACAAACTTCATCTGGCTGG; C1, 5′-GCCACCATGGGCTACCCCTACGACGTGCCCGACTACGCCAGCCTGAATCGCATCAGC; C2, 5′-GCGCTCTAGATCAGAAGGAACAATTCAGATCGCTCAGCAGGCTTGCCATCTT.

To construct β-Gal with nuclear localization signal (NLS), a polymerase chain reaction (PCR) was performed by using *Pfu* DNA polymerase (Stratagene), primers A1 and A2, and pW1, a *Lac*Z expression plasmid under the control of the CMV promoter, as a template. The resulting 900-bp PCR fragment was treated with *Not*I, and was inserted into the *Not*I-*Eco*RV site of pCANCre harboring the NLS of SV40 (a gift from I. Saito). The generated plasmid was used in the next PCR with primers A3 and A2. The resulting 900-bp fragment was digested with *Bst*BI and *Cla*I, and inserted back into pW1 (pW1n). The murine granulocyte colony-stimulating factor receptor (G-CSFR) cDNA was cut out from pLHdX-1 (a gift from S. Nagata) with *Hin*dIII and *Xba*I, and inserted into the *Hin*dIII-*Xba*I site of pUC19, which was then digested with *Bsp*120I and *Xba*I and ligated with a synthetic adapter produced by annealing equimolar amount of B1 and B2 to each other (pUGC). This adapter contains a NIa-recognition sequence (SQMKFVFQ/SD) derived from the junction between the NIb and coat protein in the ClYVV polyprotein. The 3.3-kb *Sph*I-*Bsm*I fragment derived from pW1n and 2.4-kb *Hin*dIII-*Sph*I fragment from pUGC were ligated into a CMV-expression plasmid (pGCL). A control plasmid lacking the NIa cleavage site (pGL) was constructed by digesting pGCL with *Apa*I and *Sph*I, blunt-ending, and self-circularizing. To construct NIa protease tagged with an HA epitope, PCR was conducted by using primers C1 and C2, and pTVNIa0-27 (10) as a template. To facilitate translation of the NIa protease in mammalian cells, the codon usage in primers C1 and C2 was optimized (11). The resulting 780-bp fragment was inserted into the *Sma*I site of pEB, generating pHNEB, a dicistronic expression plasmid, carrying HA-tagged NIa in the first cistron and the blasticidin resistance gene (bsr) in the second. Between the two cistrons are the internal ribosome entry site (IRES) derived from encephalomyocarditis virus. Plasmid containing HA-NIa in the opposite direction was also constructed. The luciferase gene in the first cistron has been described (12). The G-CSF receptor and β-galactosidase do not harbor a consensus sequence at which the NIa protease cuts. All the PCR-amplified DNA fragments were verified by sequencing.

Cell culture and transfection. 293 cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal calf serum and penicillin/streptomycin.

A total of 4 μg of plasmid DNA was transfected to 2×10^5 of 293 cells/well in 6-well plates by a standard calcium phosphate precipitation method. For colony formation assay, cells were replated to 6-well plates at appropriate splits 24 hr after transfection, and incubated for 10 days in the presence of 10 μg/ml blasticidin S (Funakoshi, Japan). Several colonies were picked up and expanded for further analyses.

Western blotting. Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in a lysis buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40) supplemented with 1 mM phenylmethylsulfonyl fluoride and 500 U/ml of aprotinin as described before (13). Five micrograms of lysate was subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were incubated with anti-β-gal antibody (Oncogene Science) at a dilution of 1:1000 or with 2.5 μg/ml of anti-HA antibody 12CA5 (Boehringer-Manheim, Germany) in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) for 1 hr at room temperature, and then were incubated with anti-mouse IgG labeled with horseradish peroxidase for 30 min at room temperature in TBST. Chemiluminescent signals were detected by using the ECL system (Amersham, UK).

X-gal staining and immunofluorescence analysis. For detection of β-gal enzyme activity, cells were fixed with a formaldehyde/glutaraldehyde solution and were stained with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) according to a standard protocol.

Immunofluorescence analysis was performed as described elsewhere (14). Briefly, cells were fixed with 3.5% formaldehyde in PBS on ice for 20 min, and treated with cold 70% ethanol at −20°C for 7 min, 100% ethanol for 7 min, and 70% ethanol for 5 min. They were incubated with the anti-β-gal antibody diluted 1:50, and then with FITC-conjugated anti-mouse antibody (DAKO, Denmark) diluted 1:30, each time for 30 min at room temperature. After being washed with PBS twice, the samples were observed by fluorescence microscopy.

RESULTS

Subcellular localization of chimeric protein. 293 cells were transfected with pGCL or pGL along with pHNEB or an empty vector. Figure 1B shows the expression of the chimeric proteins. Without transfection

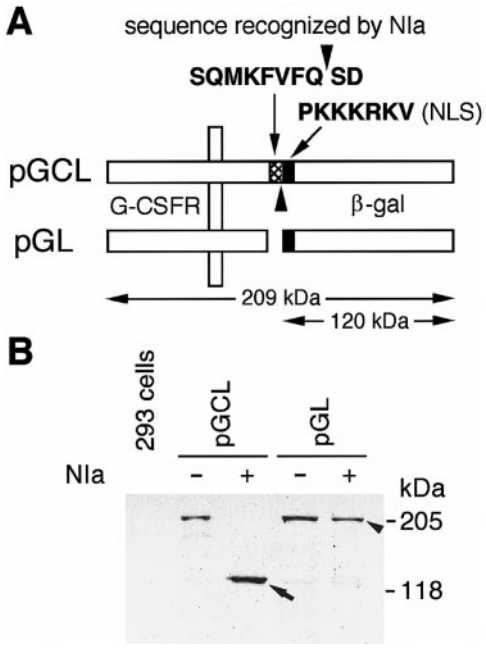

FIG. 1. (A) Schematic representation of plasmids expressing chimeric proteins. pGCL expresses a chimeric protein consisting of G-CSF receptor (G-CSFR) and β-galactosidase (β-gal) with nuclear localization signal (NLS, black box). Between the two, a NIa-recognition site, SQMKFVFQSD, is inserted (hatched box). pGL expresses a fusion protein without the NIa-protease cleavage site. Arrowhead, potential cleavage site for NIa protease. (B) Western analysis of chimeric proteins in the presence (+) or absence (−) of NIa protease, by using anti-β-gal antibody. Arrowhead, 209-kDa chimeric protein; arrow, 120-kDa cleavage product.

with pHNEB, both pGCL and pGL produced proteins of approximately 210 kDa (arrowhead) that reacted with anti-β-gal antibody, as expected. On cotransfection with pGCL and pHNEB, a band of about 120 kDa was detected (arrow), whereas transfection with pGL and pHNEB produced only 210-kDa protein. These results indicated that the chimeric protein translated from pGCL was cleaved by the NIa protease and that the protease recognized a consensus sequence between the G-CSF receptor and nβ-gal portions, because the fusion protein expressed from pGL that did not have a NIa-recognition sequence was not cleaved even when NIa was expressed. Next, to visualize the subcellular localization of chimeric proteins, transfected cells were stained with X-gal (Fig. 2A). In 293 cells transfected with pGCL or pGL alone, the cytoplasm was mainly stained, while the nuclei were translucent. On the other hand, 293 cells transfected with pGCL and pHNEB showed marked staining in the nuclei (arrowheads), whereas in cells cotransfected with pGL and pHNEB, the cytoplasmic staining pattern did not change. To confirm the localization of nβ-gal, immunofluorescence analysis was performed (Fig. 2B). As for X-gal staining, in 293 cells transfected with pGCL or pGL alone, the cytoplasm was predominantly positive for β-gal. By contrast, when cells were cotransfected with pGCL and pHNEB, the fluorescent signal was also detected in the nuclei (arrowheads).

NIa protease is not cytotoxic to 293 cells. The NIa protease recognizes only three amino acid residues at its cleave site. To examine whether cells can tolerate the overexpression of NIa protease, a colony formation assay was conducted. As shown in Fig. 3A, three plasmid DNAs harboring dicistronic genes were used in which the bsr was placed downstream of the EMCV-IRES. One harbors the NIa protease gene in the first cistron, the second also carries the NIa gene, but in the opposite orientation, and the third contains a luciferase gene in the first cistron. Following transfection to 293 cells with these three plasmids, cells were replated at a 1:500 split, and incubated further for 10 days in the presence of blasticidin. The right panel in Fig. 3A is a representative result of formed resistant colonies. The number of colonies produced was not significantly different among the three transfections, suggesting that the NIa protease is not toxic to 293 cells. Next, to confirm that stable clones expressed the NIa protease, several clones were selected and expanded. The result of analysis of a representative clone is shown in Fig. 3B and 3C. When the cells were transfected with pGCL, two proteins were detected with anti-β-gal antibody; one was approximately 210 kDa in size, and the other was 120 kDa, a result similar to Western analysis in Fig. 1B. On the other hand, when the cells were transfected with pGL, only a 210-kDa band was detected. Figure 1C shows the subcellular localization of β-gal activity as detected by X-gal staining. When using pGCL, most of the cells positive for X-gal also showed nuclear staining. In contrast, in cells transfected with pGL, the main stained site was the cytoplasm, which is also comparable to the data presented in Fig. 2A. Some deeply stained cells seem to show nuclear accumulation of β-gal, however, the positive sites in these cells were localized to the perinuclear regions.

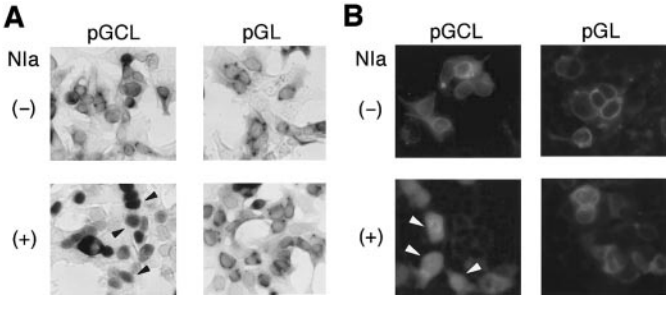

FIG. 2. Localization of β-galactosidase after transfection with pGCL or pGL along with or without NIa-expression plasmid. (A) The enzyme activity of β-gal was visualized by X-gal staining. Arrows, localized staining in the nuclei. (B) Immunofluorescence analysis for detection of β-gal protein was performed with anti-β-gal antibody. Arrows, positive signal in the nuclei.

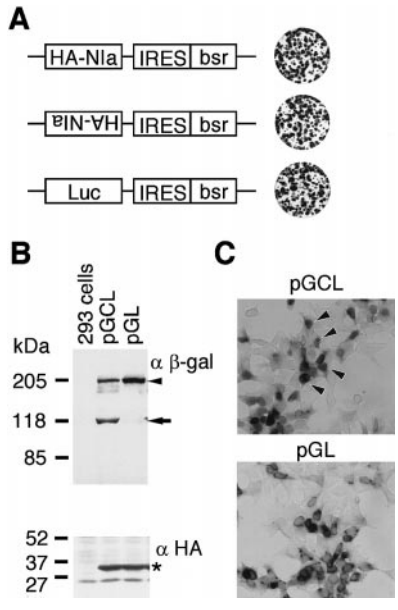

FIG. 3. (A) Colony formation assay for estimating cytotoxicity. Dicistronic plasmids expressing NIa protease tagged with an HA epitope or luciferase were transfected. A selection marker, blasticidin resistance gene (bsr), was placed downstream of the internal ribosome entry site (IRES). A plasmid with the HA-tagged NIa gene inserted in the opposite orientation was also used. The right panel is resistant colonies after a 10-day selection. (B) Expression of chimeric proteins in 293 cells stably expressing NIa protease. After transfection with pGCL or pGL, cells were lysed and subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred to PVDF membranes, and signals were detected by using anti-β-gal or anti-HA antibody. Arrowhead, intact fusion protein; arrow, cleaved product; asterisk, NIa protease. (C) Subcellular localization β-gal visualized with X-gal staining. A cell clone expressing constitutively NIa protease was transfected with pGCL or pGL. In cells transfected with pGCL, the nuclei were predominantly stained (arrowheads). In contrast, cells transfected with pGL showed localization of β-gal activity mainly in the cytoplasm.

DISCUSSION

In the present study, we explored a novel approach to the functional regulation of nuclear proteins, altering their subcellular localization. The model experiment demonstrate that a nuclear protein, β-galactosidase with NLS, fused to the transmembrane domain of a membrane receptor protein, G-CSFR, was localized exclusively in the cytoplasm as shown in Fig. 2. The chimeric protein was efficiently cleaved in 293 cells when coexpressed with the NIa protease (Fig. 1). In contrast, the fusion protein without the NIa-recognition sequence remained intact even in the presence of the NIa protease. These results indicated that the NIa protease, originally derived from a plant virus, clover yellow vein virus, specifically and efficiently cleaved at the recognition site in mammalian cells. Moreover, X-gal staining and immunofluorescence microscopy demonstrated that the β-gal portion with SV40 NLS released from the chimeric molecule was transported into the nuclei (Fig. 2). This unique switching system for nuclear proteins by fusing them to a membrane protein has not been reported.

We inserted a peptide sequence containing a NIa-cleavage site (SQMKFVFQ/SD) between G-CSFR and nβ-gal. The NIa protease used here is considered able to recognize a consensus sequence consisting of three essential amino acids, FQ/S. The substrate-specificity of the ClYVV-NIa protease seems to be relatively low. However, a mutational analysis of a cleavage site by TEV-NIa demonstrated that amino acids other than essential residues in the consensus sequence affect the cleavability (15). Moreover, the substrate specificity of plum pox potyvirus NIa protease has been shown to be influenced by amino acid residues outside the consensus sequence (16). These results suggest that the NIa protease recognizes and cleaves specifically the substrate in a more specific manner. Therefore, the proteolysis of cellular proteins by the NIa protease may be negligible.

One possible application of a system for regulating nuclear proteins by altering their subcellular localization is the establishment of stable cell lines that constitutively express exogenous cytotoxic proteins, such as adeno-associated virus (AAV) Rep proteins. Recombinant AAVs have strong potential as safe and efficient transducing vectors. At present, AAV vectors are generally produced by transient transfection on a large scale, which is tedious and time-consuming. In order to simplify the production system, packaging cell lines that do not require transfection should be established. In spite of many efforts, high-titer packaging cell lines have not been reported. Another strategy for producing AAV vectors more easily is the use of recombinant adenovirus carrying the AAV genome. However, such recombinant adenovirus has not been rescued yet, partly because the Rep proteins suppress the replication of adenovirus. In an attempt to establish a packaging cell line for AAV vector production, we are currently testing the system presented here to regulate the Rep proteins. A preliminary data indicate that a cell line constitutively expressing the cytotoxic Rep proteins fused to the G-CSFR could be established. The cell line inducibly produced AAV vectors by the expression of NIa-protease, suggesting that the chimeric Rep proteins forced to remain in the cytoplasm were not functional as well as not cytotoxic, and that the cleaved Rep proteins were transported to the nuclei and functioned normally. The finding clearly confirms the notion that proteins must be located in the proper site in cells to exhibit their intrinsic functions. The present study also suggests that a novel strategy to regulate the subcellular localization of nuclear proteins by the use of a viral protease would be able to apply to other nuclear proteins.

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