Regulation of nuclear import by light-induced activation of caged nuclear localization signal in living cells

Yoriko Watai, Ichiro Sase*, Hirofumi Shiono[1], Yoshitaro Nakano[2]

Laboratory of Molecular BioPhotonics, 5000 Hamakita, Shizuoka 434-8555, Japan

Received 6 November 2000; accepted 7 December 2000

First published online 21 December 2000

Edited by Felix Wieland

Abstract A novel fluorescence probe suitable for the study of nuclear import in living cells has been developed. The lysine-128 residue in SV40 T-antigen nuclear localization signal (NLS) was converted to a caged lysine with the amino acid blocked by a photocleavable protecting group. Following irradiation of ultraviolet (UV) light, the caged NLS conjugate translocated into and accumulated in the nucleus within 20 min similar to uncaged NLS conjugate. Maximum import rate saturated approximately 4.78 ± 0.21% per minute when the duration of irradiation was more than 1/15 s (22 mW/cm^2). Caged NLS conjugate tended to distribute near the surface of the nucleus, and this association became stronger after UV irradiation. The caged conjugate enabled us to regulate the initial state of the reaction, both spatially and temporally.

Key words: Caged nuclear localization signal; Light-induced activation; Nuclear import; Cy7

1. Introduction

In eukaryotic cells, the cytoplasm and the nucleus are separated by the nuclear envelope. Nuclear proteins are synthesized in the cytoplasm and imported into the nucleus through the nuclear pore complex (NPC) located on the nuclear envelope [1]. Most of nuclear protein import is mediated by consensus sequence of basic amino acids, called nuclear localization signal (NLS), which interacts with two essential components referred to as importin α/β [2–7]. Different types of NLS factors and their structures have been identified in several years [8–13]. Mechanisms of nuclear transport have been studied recently using in vivo and in vitro techniques [14–25]. However, there have been only a few studies concerned with the real-time kinetics of cytoplasm–nucleus transport in living cells. For real-time observation in living cells, the development of a controllable and sensitive (highly detectable) probe is required.

Caged compounds are inactivated biological molecules with reaction site(s) blocked by a photocleavable protecting group. The biological activity of caged compounds can be controlled by irradiating photolytic light, usually ultraviolet (UV) light of approximately 350 nm. Since light irradiation is easily controllable in terms of time, area, and intensity, caged compounds have been used to investigate the molecular basis of intracellular processes [26–28]. The caged molecules used in most studies are small molecules such as caged glutamate, Ca^{2+} and ATP. In spite of the obvious potential of this technique, only a few studies using caged peptides or proteins have been reported, due to difficulties in preparing caged compounds with large molecular weights [29,30]. In this study, a method of solid-phase peptide synthesis using caged amino acid [31–33] was utilized, demonstrating the simplicity and adaptability of introducing caged amino acid in various positions within the peptide sequence.

Cellular autofluorescence is often a limiting factor when attempting to achieve high fluorescence detectability in vivo. Increasing excitation wavelengths have been proposed to decrease the intensity of autofluorescence (Sase, in preparation). Cy7 was therefore used to overcome this problem [34–36]. The properties of Cy7 include (1) high excitation and emission wavelengths (ex. 750 nm, em. 776 nm), (2) a high extinction coefficient (250 000 M^{-1} m^{-1}), and (3) relatively narrow excitation and emission bands [35]. Caged compounds are also stable in the infrared–red spectral range, whereas photolysis occurs by UV light (excitation light is low enough that the effect of two-photon excitation is negligible).

In this report we describe a method for studying nuclear import in living cells with a novel caged NLS. Caged NLS conjugated to Cy7 labeled bovine serum albumin (BSA) was microinjected into living cells, and the time course of nuclear import was detected in real-time. With this caged system, changes in the intracellular distribution of the NLS labeled molecule had been monitored from the steady state. In addition the quantity of released conjugate can be controlled in vivo by controlling the level of UV irradiation. This new conjugate is thus useful for exploring the kinetics of biological molecules in living cells.

2. Materials and methods

2.1. Cell culture

HeLa cells (RCB007; Riken Cell Bank) were cultured in minimum essential medium (Gibco BRL) supplemented with 10% fetal bovine

*Corresponding author. Present address: Ministry of Posts and Telecommunications, Kansai Advanced Research Center, Iwaoka 588-2, Nishi-ku, Kobe, Hyogo 651-2492, Japan.
Fax: (81)-78-969 2279. E-mail: sase@po.crl.go.jp

[1] Present address: Nikon Corporation, 6-3, Nishi-ohi, 1-chome, Shinagawa-ku, Tokyo 140-8601, Japan.
[2] Present address: Nikon Corporation Instruments Company, 471, Nagaodai-machi, Sakae-ku, Yokohama, Kanagawa 244-8533, Japan.

Abbreviations: BSA, bovine serum albumin; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; NLS, nuclear localization signal

0014-5793/01/$20.00
PII: S0014-5793(00)02399-1

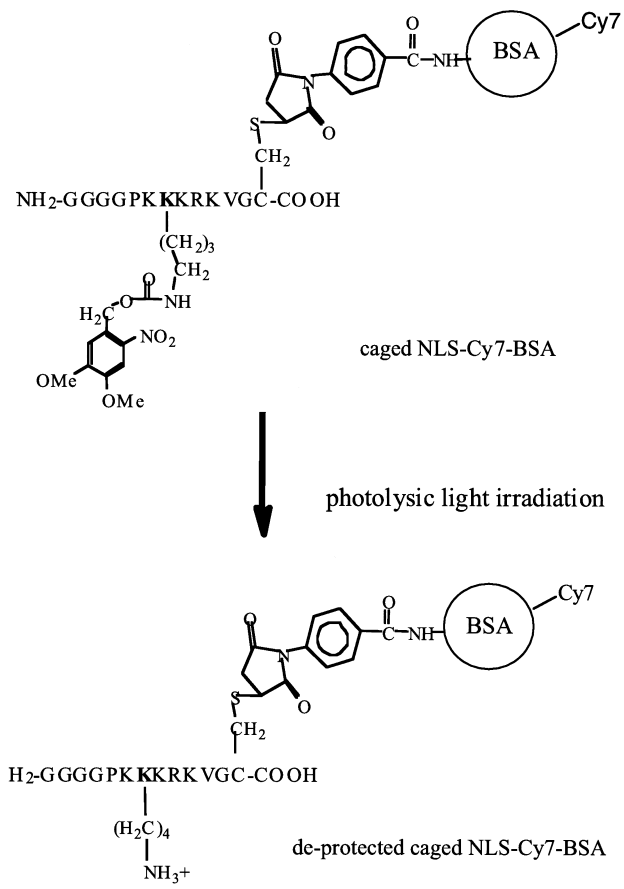

Fig. 1. De-protection model of caged NLS by photolythic light irradiation. Caged NLS, in which lysine-128 of SV40 T-antigen was converted to caged lysine, was conjugated to Cy7 labeled BSA. Following irradiation, photocleavage of the protecting group (*o*-nitrobenzyl group) takes place, and the amino acid sequence can now be recognized as the NLS sequence, PKKKRK.

serum (Gibco BRL) in a water-saturated atmosphere of 5% CO_2 at 37°C.

For microinjection, cells were seeded onto glass-bottomed 35 mm culture dishes (MatTek Co.) and cultured for 48–72 h before use.

2.2. Synthetic peptides

Caged NLS peptide with lysine-128 from SV40 T-antigen converted to caged lysine (GGGPKcagedKKRKVGGGC) (Mw 1567.8) was synthesized on an automated solid-phase peptide synthesizer using Fmoc-caged-Lys, which was obtained from a reaction of Fmoc-lys with 4,5-dimethoxy-2-nitrobenzylcarbonyl chloride (Aldrich). Synthetic peptides containing the SV40 T-antigen NLS (GGGPKKKRKVGGGC) (Mw 1327.9) and import-deficient mutated NLS (GGGPKNKRKVGC) (Mw 1256.7) named xNLS were synthesized by Sawaday Technology Co. (Tokyo, Japan).

2.3. Preparation of NLS-conjugated BSA

175 μM BSA (Mw 67,000) (Sigma) in 10 mM phosphate buffer, pH 7.5 plus 150 mM NaCl, 400 μM Cy7-Osu bifunctional reactive dye (Mw 1001.2) (Amersham Pharmacia Biotech.) and 6.2 mM of maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Mw 314.2) (Pierce) dissolved in dimethyl sulfoxide were mixed immediately and incubated for 1.5 h at room temperature. One-tenth volume of 20% methyl ammonium chloride was added during the last 10 min to stop the succinimide reaction. The reaction mixture was centrifuged with a desktop centrifuge for 2 min and the supernatant was filtered through a Sephadex G-25 column with 10 mM phosphate buffer, pH 7.0. Immediately following gel filtration, 2.4 mM caged NLS, 1.7 mM NLS, or 2.2 mM xNLS was mixed with the elute and incubated in the dark at 4°C for 12 h. Each reaction mixture was concentrated, desalted, and small molecules (i.e. Cy7, MBS, and unreacted peptides) were removed with a Microcon 30 < MW 30 000 pass filter (Millipore). BSA concentration was measured with a protein assay kit (Bio-Rad). The Cy7 labeling ratio was estimated from the extinction coefficient, 250 000 M^{-1} cm^{-1} for Cy7 [36]. The efficiency of peptide conjugation was estimated by mobility shift assay on sodium dodecyl sulfate (SDS)–polyacrylamide gels and by isoelectric focusing gel electrophoresis.

2.4. Microinjection and photolysis

To observe nuclear import of peptide-conjugated Cy7-BSA, microinjection was performed. Cultured cells seeded on glass-bottomed dishes were washed, and the medium was changed to Hanks' balanced salt solution (Gibco BRL). The glass-bottom dish was kept on the microscope at 37°C throughout the experiment by a thermo-controller. The peptide-conjugated Cy7-BSA was microinjected into the cell cytoplasm with a micromanipulator and transinjector system (Eppendorf).

Cells were irradiated with UV light 10 min after microinjection to exclude damaged cells and to allow the cells to stabilize after microinjection. A schematic of the photolytic reaction of caged NLS by

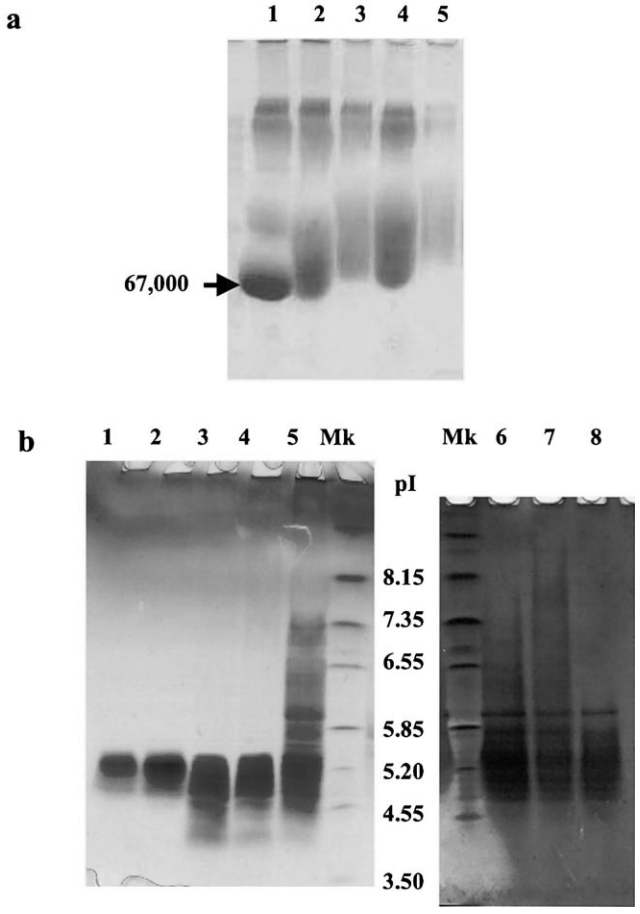

Fig. 2. Mobility shift assay of synthetic peptides conjugated to Cy7 labeled BSA. BSA was conjugated to synthetic peptides using the hetero-functional crosslinker MBS. Samples were electrophoresed on 8% SDS–polyacrylamide gels or 4% polyacrylamide isoelectric focusing gels. Samples were stained with Coomassie brilliant blue after electrophoreses. a: SDS–polyacrylamide gel of BSA and peptides conjugated to BSA. Lane 1, BSA; 2, MBS-modified Cy7 labeled BSA; 3, NLS-Cy7-BSA; 4, xNLS-Cy7-BSA; 5, caged NLS-Cy7-BSA. b: Isoelectric focusing gels. Lane 1, BSA; 2, Cy7 labeled BSA; 3, MBS-modified BSA; 4, MBS-modified Cy7 labeled BSA; 5, 6, NLS-Cy7-BSA (independent preparation); 7, xNLS-Cy7-BSA; 8, caged NLS-Cy7-BSA; Mk, p*I* marker proteins (Broad p*I* calibration kit (pH 3–10), Amersham Pharmacia Biotech.).

irradiation is shown in Fig. 1. The caged compounds of which maximum photolytic reaction occurs at 350 nm were used.

2.5. Microscope and photolysis system

A confocal laser scanning microscope system for near infrared–red was developed in our laboratory (Sase, in preparation). Nuclear import was observed using Cy7 excited by a laser diode (LD) (680 nm, 30 mW) via an objective lens (PlanApo60 WI, N.A. 1.20, Nikon) attached to an inverted microscope (TE-300, Nikon). Fluorescence signal was detected with a −30°C perutie cooled GaAs photomultiplier (Hamamatsu). Fluorescence intensities of the nuclear and cytoplasmic areas in each cell were quantified using an Argus-1000 image

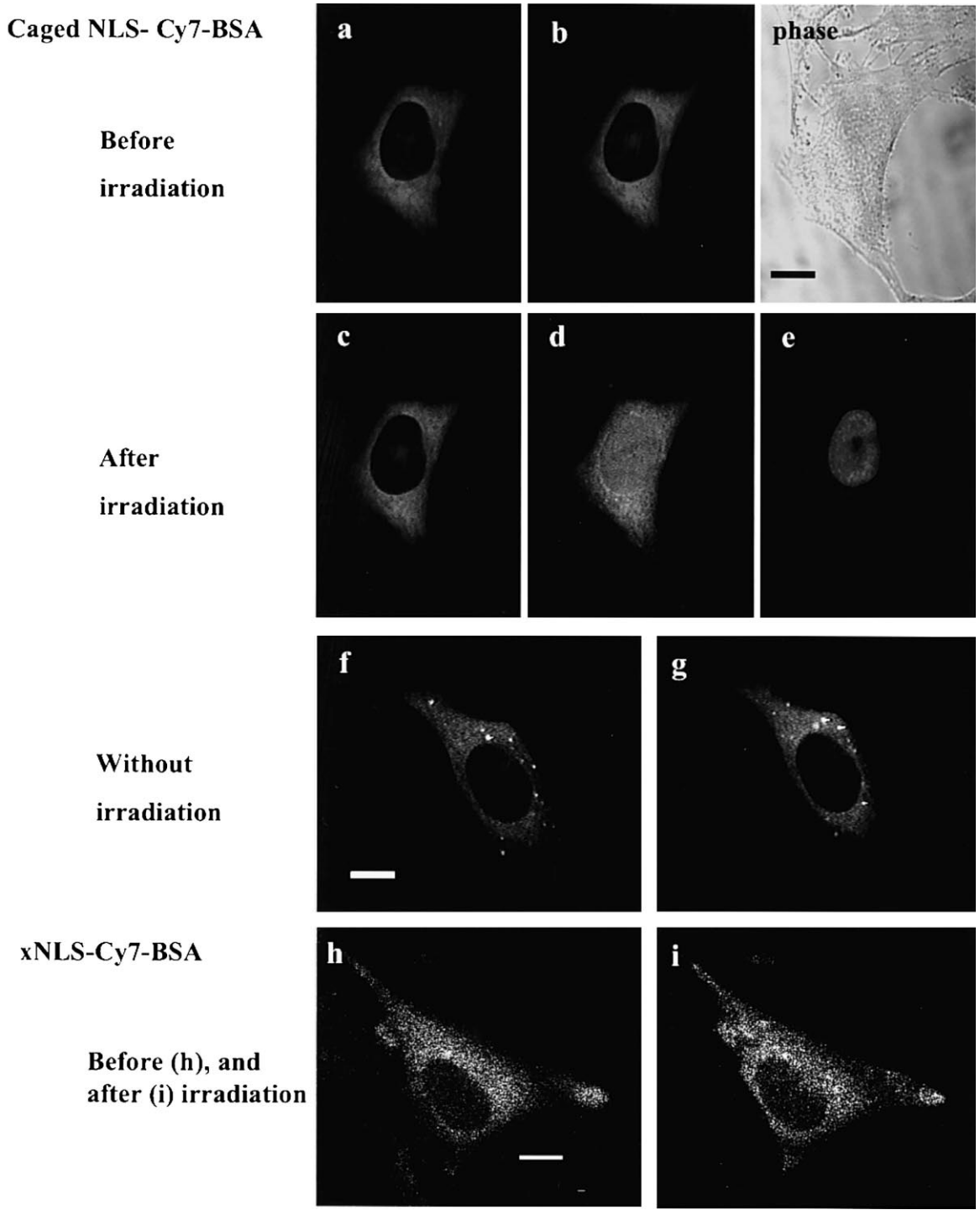

Fig. 3. Fluorescence images of caged NLS-Cy7-BSA and xNLS-Cy7-BSA injected into the cytoplasm of HeLa cells. Injected cells were incubated at 37°C on the microscope stage. 10 min after microinjection, UV light was irradiated for 0.5 s (a–e). Nuclear accumulation of NLS-Cy7-BSA after UV irradiation is shown (a–e). a: 1 min after injection. b: 10 min after injection (just before irradiation). c: 10 s after irradiation (10.2 min after injection). d: 10 min after irradiation (20 min after injection). e: 20 min after irradiation (30 min after injection). The phase image was taken 25 min after the irradiation. f,g: Fluorescence images of non-UV-irradiated caged NLS-Cy7-BSA. Images were taken 10 min (f) and 40 min (g) after the injection. h,i: Fluorescence images of microinjected xNLS-Cy7-BSA. Images were taken 10 min after injection (just before irradiation) (h) and 30 min after irradiation (40 min after injection) (i). Scale bar, 20 μm.

processor (Hamamatsu), and the nucleus/whole cell ratio was calculated. A 100 W Hg lamp (Ushio) was used as the UV light source, and the irradiation time was controlled by a mechanical shutter (Sigma KOKI). Light intensities of the LD and the Hg lamp were measured beyond the objective lens with a powermeter (Advantest).

3. Results

Synthetic peptides were coupled to Cy7 labeled BSA with the crosslinker MBS. Unreacted Cy7, MBS, and excess peptide were removed by a membrane filter. To calculate the ratio of Cy7 labeled to unlabeled peptide-conjugated BSA preparation, concentrations of BSA and Cy7 were measured by protein assay kit and absorption spectra, respectively. The ratios of labeled caged NLS-Cy7-BSA, NLS-Cy7-BSA and xNLS-Cy7-BSA were 62%, 74%, and 80%, respectively. The number of peptides conjugated to BSA was estimated to be approximately 2–5 peptides per molecule according to mobility shift on SDS–polyacrylamide and isoelectric focusing gels (Fig. 2a,b). The final molecular ratio was calculated as NLS:Cy7:BSA = 2–5:0.6–0.8:1. To check the reactivity of the NLS sequence in the conjugate, both NLS-Cy7-BSA and mutant xNLS-Cy7-BSA were microinjected into cultured HeLa cells. Both conjugates diffused uniformly into the cytoplasm. However, NLS-Cy7-BSA was imported into the nucleus within 20 min, and $>80\%$ of xNLS-Cy7-BSA was retained in the cytoplasm even 30 min after the microinjection (data not shown).

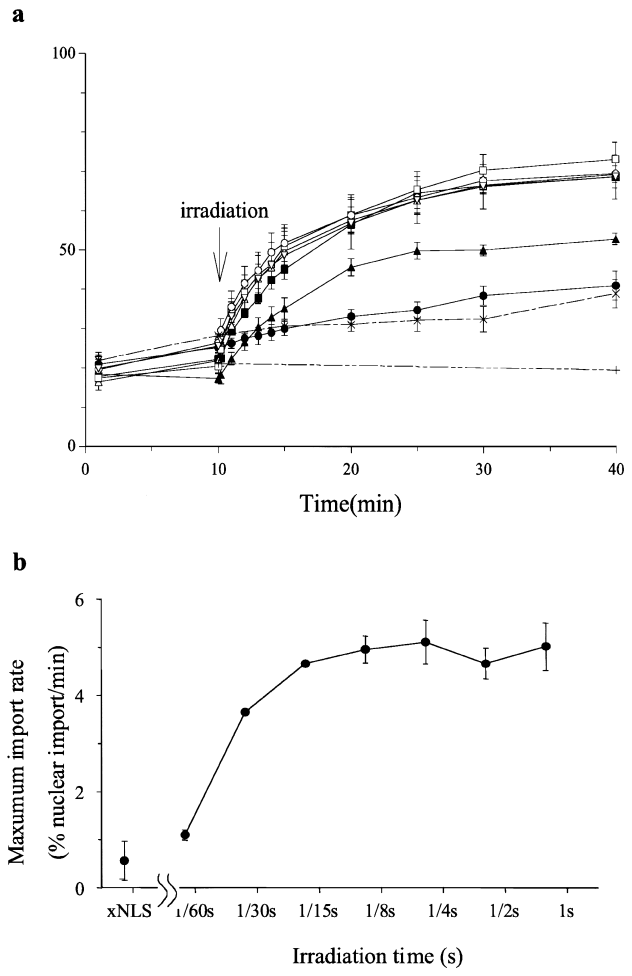

Fig. 4. Nuclear import kinetics of caged NLS-Cy7-BSA in vivo, after varying irradiation periods. Caged NLS-Cy7-BSA was microinjected into the cytoplasm of HeLa cells. Injected cells were incubated at 37°C throughout the experiments, and UV light was irradiated 10 min after microinjection. Images were acquired at different time points after microinjection, and fluorescence intensities in the nucleus and the cytoplasm were measured. a: Nucleus/whole cell ratios of fluorescence plotted as a function of time after microinjection. Each plot respects the average of 10 cells ± S.D. Caged NLS-Cy7-BSA in the absence of irradiation (+) and xNLS-Cy7-BSA (×) are plotted as broken lines. Irradiation time for each continuous line is 1/60 s (●), 1/30 s (▲), 1/15 s (■), 1/8 s (△), 1/4 s (□), 1/2 s (○) and 1 s (▽). b: The increase in nuclear import rate in the linear phase of a. Maximum nuclear import rate per min is plotted as a function of irradiation time, with that of xNLS-Cy7-BSA shown as a reference.

To examine the recovery of nuclear import ability of the probe, UV light was irradiated onto the cell body 10 min after microinjection of caged NLS-Cy7-BSA. A time course study showed that fluorescence intensity in the cytoplasm decreased whereas signal in the nucleus increased after irradiation (Fig. 3a–e). This translocation did not occur for at least 30 min in the absence of irradiation (Fig. 3f,g), and in the case of xNLS-Cy7-BSA even after irradiation (Fig. 3h,i). Nuclear import was observed even when irradiation was limited to only one-third of the area of the cytoplasm, though translocation was significantly lower (data not shown).

We assumed that the photolytic efficiency of caged NLS is dependent upon the irradiation intensity and duration, and that the kinetics of nuclear import depend on the amount of activated NLS. To investigate the quantitative effect of photoreleased conjugate in vivo, the fluorescence intensities of the nucleus and the cytoplasm in each cell were measured after various periods of UV irradiation. For quantitative measurement of fluorescence intensity, the excitation intensity of the LD laser was fixed to 0.6 mW in the sample plane (the scanning area was 200×200 μm^2, and the scanning speed was 1 s/frame). The intensity of the photolytic Hg lamp was 22 mW/cm^2 in the sample plane. Nucleus/whole cell fluorescence ratios after varying irradiation periods are plotted as a function of time after injection in Fig. 4a. Fig. 4b shows the maximum nuclear import rate as a function of irradiation time. Irradiation was performed 10 min after injection. Nuclear import of light-activated caged NLS-Cy7-BSA saturated within 20 min after irradiation. With a short irradiation period of less than 1/15 s, the nuclear import rate decreased with the decrease in duration. At irradiation periods from 1/15 s to 1 s, nuclear import was similar, the maximum import rate was 4.56–5.00% per minute ($4.78\pm0.21\%$, mean ± S.D. ($n=5$ under each condition)), and translocation was saturated at a level of 70%.

4. Discussion

Non-invasive regulation of molecular activity in living cells is an indispensable technique for understanding biological mechanisms at the cellular level. To remotely activate intracellular import in living cells, we synthesized a novel biological conjugate using a caged peptide. We also assembled an infrared–red confocal system for the remote activation and imaging of conjugated molecules in living cells. Caged conjugates enabled us to regulate the initial state of the reaction spatially and temporally.

The development of an in vitro system that mimics the

transport of proteins from the cytoplasm into the nucleus (e.g. digitonin-treated cells) has allowed the characterization of a number of proteins involved in this process [17–19]. To understand the intracellular behavior of the molecules in vivo, microinjection has been used in many experimental systems under microscopy. Since microinjection involves a micropipette to introduce molecules that are already active, it is difficult to (1) regulate the spatial distribution of molecules inside of the cell, since the reaction starts immediately after microinjection, before the molecules equilibrate, and (2) to visualize the reaction of numerous cells simultaneously. In addition, cells at different stages often have to be fixed (e.g. with formaldehyde or acetone) for microscopic observation. To control the molecular import in vivo, we synthesized a caged NLS conjugate in which a single peptide in the NLS sequence (Lys-128) was caged with 2-nitrobenzyl so that recognition of the sequence by other proteins was inhibited in the cell. For synthesis, we used an automated solid-phase peptide synthesizer with Fmoc-caged-Lys. This general synthetic system expands the versatility of the system for application to other bio-functional peptides. Synthesized caged conjugate was stable enough that even after purification of caged NLS conjugate, caged Lys remained protected at a high rate ($>95\%$). In addition, unexpected photolysis was negligible even with irradiation at 680 nm for Cy7 excitation.

Microinjected caged NLS conjugate spread throughout the cytoplasm and was stable for more than 1 h under the microscope. Cells did not show any morphological changes (e.g. shrinkage or detachment from the coverglass) after microinjection or UV irradiation. After UV irradiation, caged NLS conjugate translocated smoothly into the nucleus. Considering the absorption spectrum of the nitrobenzylcarbonyl group, the photolytic efficiency, and the through-rate of the optical path, the 365 nm line spectrum of the mercury lamp was used for photolysis. The half lifetime of intermediate products during photolysis was approximately 6.5 μs [32], which is fast enough that it did not become a rate-limiting step in molecular import. To reduce damage due to microinjection, a 10 min interval was allowed between microinjection and UV irradiation, which also provided time for equilibration of the spatial distribution of conjugate inside the cytoplasm. We believe this time was important for the observation of transient reactions in vivo. For real-time tracking of the caged NLS conjugate, the infrared–red fluorophore Cy7 was used. Cultured cells often display strong autofluorescence, especially in the cytosol, such that quantitative discussion of fluorescence intensity requires precaution. With infrared–red excitation, however, autofluorescence is negligible, and quantitative analysis is possible even of the cytosol.

The speed of nuclear import of the NLS conjugate is reported to increase with the amount of bound NLS peptide [24]. The number of bound NLS peptides per molecule in this study was calculated as 2–5, and the time course for import was similar to that determined by Tachibana et al. [24]. This, along with the finding that photoreleased NLS conjugate was imported into the nucleus at the same speed as non-caged NLS conjugate (data not shown), provides evidence that caging does not affect the recognition of the NLS sequence. Maximum import rate (V_{max}) was saturated at approximately $4.78 \pm 0.21\%$ per minute when the period of irradiation was more than 1/15 s, which was calculated as 6000–7000 molecules/s.

Careful observation of the caged NLS conjugate inside the cells showed caged NLS conjugate tended to distribute strongly near the surface of the nucleus, and this tendency increased after UV irradiation. This unique distribution was not observed by microinjection of BSA-Cy7 conjugate alone; it was observed to some extent by microinjection of xNLS conjugate. This implies that caged NLS conjugate may interact weakly with importin (or importins) before photocleavage and aggregate around the nucleus, which leads to interaction with Nup358 of the NPC [37–39]. An increase in fluorescence signal near the surface of the nucleus was not observed when the number of injected molecules was low, in agreement with the fact that the nuclear import rate decreased as the irradiation time decreased (decrease in the number of photoreleased molecules). When the irradiation period was less than 1/15 s, import rate was not strictly proportional to irradiation time, due to variance in the microinjection volume. If the assembly of caged NLS conjugate is due to interaction with importins, it implies that the translocation of the conjugate requires at least two steps before entering the NPC. It is possible that only one of the importins, α or β, is bound to the conjugate and waiting for the other to bind. To examine this, it would be necessary to visualize each protein using double-label fluorescence or introduce GFP. Peptide sequence recognition plays an important role in signal transduction in many cellular systems. The caged NLS conjugate will be useful not only for the regulation of molecular import but also for the study of photo modulation of other proteins inside the cell. There are less toxic caged compounds available for use in vivo [40]. When applying recently developed single molecule imaging techniques in vivo, the use of infrared–red excitation fluorophores as molecular tags will reduce cellular autofluorescence and also broaden the spectral range for fluorophore.

Acknowledgements: We thank Mr. H. Terada and Dr. M. Hirano (Hamamatsu Photonics K.K., Shizuoka, Japan) for adjustment of the IR confocal laser scanning microscope. We also thank Drs. H. Matsumoto (Hamamatsu Photonics K.K.), T. Suga (Nikon, Tokyo, Japan) and Ms. M. Takayanagi (Olinova, Shizuoka, Japan) for helpful advice throughout these experiments.

References

[1] Turpin, P., Ossareh-Nazari, B. and Dargemont, C. (1999) FEBS Lett. 452, 82–86.
[2] Lanford, R., Kanda, P. and Kennedy, R.C. (1986) Cell 46, 575–582.
[3] Dingwall, C., Sharnick, S.V. and Laskey, R.A. (1982) Cell 30, 449–458.
[4] Görlich, D., Vogel, F., Mills, A.D., Hartman, E. and Laskey, R.A. (1995) Nature 377, 246–248.
[5] Chi, N.C., Adam, E.J. and Adam, S.A. (1995) J. Cell Biol. 130, 265–274.
[6] Imamoto, N., Shimamoto, T., Kose, S., Takao, T., Tachibana, T., Matsubae, M., Sekimoto, T., Shimonishi, Y. and Yoneda, Y. (1995) FEBS Lett. 368, 415–419.
[7] Moroianu, J., Blobel, G. and Radu, A. (1995) Proc. Natl. Acad. Sci. USA 92, 2008–2011.
[8] Boulikas, T. (1993) Crit. Rev. Eukaryot. Gene Expr. 3, 193–227.
[9] Siomi, H. and Dreyfuss, G. (1995) J. Cell Biol. 129, 551–560.
[10] Schlenstedt, G. (1996) FEBS Lett. 389, 75–79.
[11] Yoneda, Y. (1997) J. Biochem. 121, 811–817.
[12] Conti, E., Uy, M., Leighton, L., Blobel, G. and Kuriyan, J. (1998) Cell 94, 193–204.
[13] Cingolani, G., Petosa, C., Weis, K. and Müller, C.W. (1999) Nature 399, 221–229.
[14] Görlich, D. and Mattaj, I.W. (1996) Science 271, 1513–1518.

[15] Nigg, E.A. (1997) Nature 386, 779–787.
[16] Moroianu, J. (1998) J. Cell. Biochem. 70, 231–239.
[17] Newport, J. (1987) Cell 48, 205–217.
[18] Adam, S.A., Sterne-Marr, R. and Gerace, L. (1990) J. Cell. Biol. 111, 807–816.
[19] Cserpán, I. and Udvardy, A. (1995) J. Cell. Sci. 108, 1849–1861.
[20] Adam, E.J. and Adam, S.A. (1994) J. Cell Biol. 125, 547–555.
[21] Kehlenbach, R.H., Dickmanns, A. and Gerace, L. (1998) J. Cell Biol. 141, 863–874.
[22] Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M., Nomura, M. and Goldfarb, D.S. (1996) J. Cell Biol. 135, 329–339.
[23] Percipalle, P., Jonathan, P., Butler, G., Finch, J.T., Jans, D.A. and Rhodes, D. (1999) J. Mol. Biol. 292, 263–273.
[24] Tachibana, T., Hieda, M. and Yoneda, Y. (1999) FEBS Lett. 442, 235–240.
[25] Rosorius, O., Heger, P., Stelz, G., Hirschmann, N., Hauber, J. and Stauber, R.H. (1999) BioTechniques 27, 350–355.
[26] Marriot, G. (1994) Biochemistry 33, 9092–9097.
[27] Sakurai, T., Terakawa, S., Yamamoto, S., Koshimoto, H. and Watanabe, A. (1999) in: Slow Synaptic Responses and Modulation (Kuba, K., Higashida, H., Brown, D.A. and Yoshioka, T., Eds.), pp. 361–366, Springer-Verlag, Tokyo.
[28] Suga, T., Hirano, M., Takayanagi, M., Koshimoto, H. and Watanabe, A. (1998) Biochem. Biophys. Res. Commun. 253, 423–430.
[29] Bayley, H., Chang, C., Miller, W.T., Niblack, B. and Pan, P. (1998) Methods Enzymol. 291, 117–135.
[30] Marriot, G. and Walker, J.W. (1999) Trends Plant Sci. 4, 330–334.
[31] Haridasan, V.K. and Pillai, V.N. (1987) Proc. Indian Sci. Acad. 53, 717–728.
[32] Tatsu, Y., Shigeri, Y., Sogabe, S., Yumoto, N. and Yoshikawa, S. (1996) Biochem. Biophys. Res. Commun. 227, 688–693.
[33] Tatsu, Y., Shigeri, Y., Ishida, A., Kameshita, I., Fujisawa, H. and Yumoto, N. (1999) Bioorg. Med. Chem. Lett. 9, 1093–1096.
[34] Southwick, P.L., Ernst, L.A., Tauriello, E.W., Parker, S.R., Mujumdar, R.B., Mujumdar, S.R., Clever, H.A. and Waggoner, A.S. (1990) Cytometry 11, 418–430.
[35] Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J. and Waggoner, A.S. (1993) Bioconjug. Chem. 4, 105–111.
[36] Roederer, M., Kantor, A.B., Parks, D.R. and Harzenberg, L.A. (1996) Cytometry 24, 191–197.
[37] Allen, T.D., Cronshaw, J.M., Bagley, S., Kiseleva, E. and Goldberg, M.W. (2000) J. Cell Sci. 113, 1651–1659.
[38] Ryan, K.J. and Wente, S.R. (2000) Curr. Opin. Cell Biol. 12, 361–371.
[39] Talcott, B. and Moore, M.S. (1999) Trends Cell Biol. 9, 312–318.
[40] Shiono, H., Watanabe, A. and Nohta, H. (1999) Neurosci. Res. 23, S52.