A photogenerated pore-forming protein

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Background: The permeabilization of cells with bacterial pore-forming proteins is an important technique in cell biology that allows the exchange of small reagents into the cytoplasm of a cell. Another notable technology is the use of caged molecules whose activities are blocked by addition of photoremovable protecting groups. This allows the photogeneration of reagents on or in cells with spatial and temporal control. Here, we combine these approaches to produce a caged pore-forming protein for the controlled permeabilization of cells.

Results: 2-Bromo-2-(2-nitrophenyl)acetic acid (BNPA), a water-soluble cysteine-directed reagent for caging peptides and proteins with the α-carboxy-2-nitrobenzyl (CNB) protecting group, was synthesized. Glutathione (γ-Glu-Cys-Gly) was released in high yield from γ-Glu-CysCNB-Gly by irradiation at 300 nm. Based on this finding, scanning mutagenesis was used to find a single-cysteine mutant of the pore-forming protein staphylococcal α-hemolysin (αHL) suitable for caging. When αHL-R104C was derivatized with BNPA, pore-forming activity toward rabbit erythrocytes was lost. Near UV irradiation led to regeneration of the cysteine sulfhydryl group and the restoration of pore-forming activity.

Conclusions: Caged pore-forming proteins are potentially useful for permeabilizing one cell in a collection of cells or one region of the plasma membrane of a single cell. Therefore, αHL-R104C-CNB and other caged proteins designed to create pores of various diameters should be useful for many purposes. For example, the ability to introduce reagents into one cell of a network or into one region of a single cell could be used in studies of neuronal modulation. Further, BNPA should be generally useful for caging cysteine-containing peptides and single-cysteine mutant proteins to study, for example, cell signaling or structural changes in proteins.

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Introduction

Pore-forming proteins such as bacterial exotoxins have been widely used to permeabilize cells for experimental biology [1,2]. They may also have applications in biotechnology, for example as components of sensors or in drug delivery [3,4]. Pore-forming proteins would be even more useful if their activities could be controlled by external stimuli. With this in mind, we have been using genetic engineering and targeted chemical modification to introduce triggers and switches into such proteins. Our principal target has been staphylococcal α-hemolysin [5].

Staphylococcal α-hemolysin

α-Hemolysin (αHL, also known as α-toxin) is secreted by *Staphylococcus aureus* as a 293 amino acid [6], water-soluble, monomeric [7] polypeptide. Seven copies of this monomer assemble to form a heptameric pore [8] that allows molecules of up to 3 kD to pass through lipid bilayers [9–11]. Therefore, αHL can be extremely useful for introducing small molecules into cells without releasing macromolecules and organelles [2]. While αHL can self-assemble in the presence of pure lipid bilayers [12] or the detergent deoxycholic acid [7], assembly is promoted by receptors that are present on certain cells [5], such as the rabbit erythrocytes (rRBC) used in the present study [13,14].

α-Hemolysin with engineered triggers and switches

The αHL polypeptide can be thought of as two domains connected by a glycine-rich central loop [15]. The loop is highly susceptible to proteolytic digestion when the monomer is in solution, but becomes occluded during assembly [15–17]. Recent findings suggest that the loop lines part of the lumen of the transmembrane channel in the fully assembled pore [18,19]. By mutagenesis of this crucial region of αHL, we have been able to produce variants that are activated by proteolytic digestion [20] or turned on and off by EDTA and divalent metal ions, respectively [19]. The protease-activated trigger was generated through molecular complementation mutagenesis [21]. Constructs were made that comprised the αHL monomer in two parts, containing a sequence overlap in the loop region. When the redundant piece of polypeptide chain was removed by site-specific proteolysis, an active two-chain molecule, nicked in the loop, was obtained [20]. The metal-actuated switch was made by replacing five residues in the center of the loop with a pentahistidine motif [19]. This variant of αHL is inactivated by divalent metal ions, such as Zn(II), that can be coordinated by imidazole rings. Activity is restored by chelators, such as EDTA. The action of Zn(II) is twofold: it can both prevent a late step in assembly (conversion of a heptameric prepore to the functional pore) [17], and block the fully assembled pore [19].

Triggering and switching the activities of proteins with biochemical, chemical and physical stimuli

Protease treatment can be thought of as a biochemical 'stimulus' and it should, in principle, also be possible to

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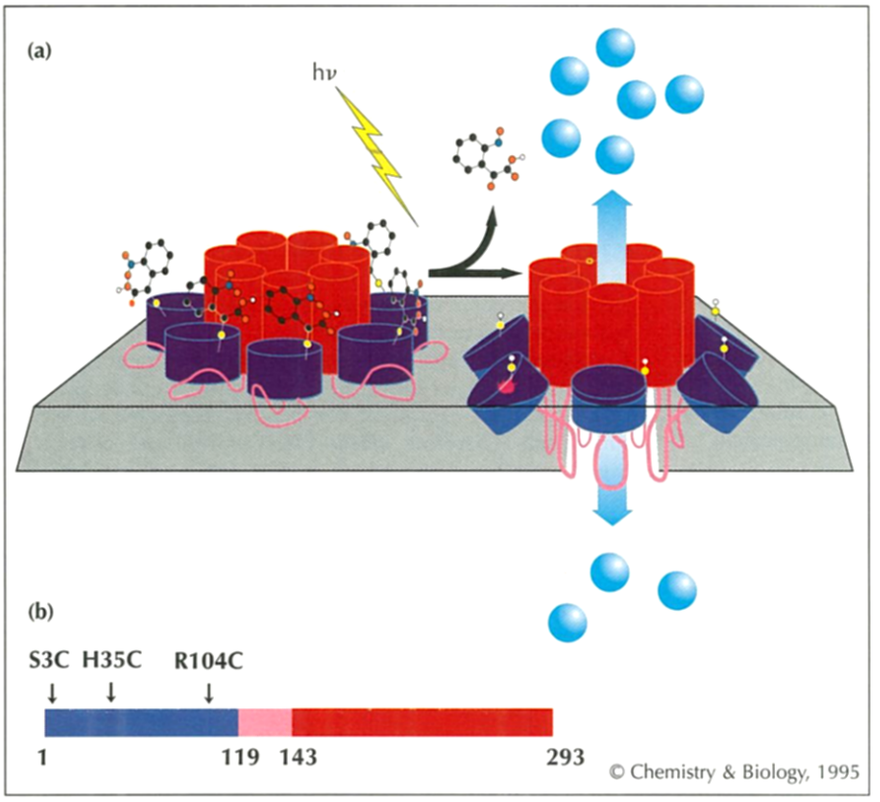

Fig. 1. Design of a photoactivated pore-forming protein. **(a)** The final step in the assembly of α-hemolysin involves the conversion of an inactive heptameric 'prepore' to a structure in which the central, glycine-rich loop (pink) is thought to line a segment of the transmembrane pore. We sought to find a single-cysteine mutant of αHL that could be inactivated with a photoremovable protecting group (shown out of scale here). Upon photolysis, the uncaged αHL would penetrate the membrane to form functional pores. **(b)** Positions of amino-acid replacements in single-cysteine mutants of αHL used in this work: S3C (Ser3→Cys), H35C (His35→Cys) and R104C (Arg104→Cys). All are located in the amino-terminal domain (blue) which is separated from the carboxy-terminal domain (red) by the central loop (pink).

control activation by using other enzymes such as protein kinases and phosphatases (see [22]). Divalent metal-ion modulation is a chemical 'stimulus' that has also been used to switch enzymes on and off [23,24]. Covalent chemical modification has also been used to activate mutant proteins, including enzymes [25] and membrane proteins [26,27]. Recently, we showed that a single-cysteine mutant of αHL, αHL-H35C, can be activated by iodoacetamide [28]. Presumably, the alkylation product, S-carboxamidocysteine, mimics the necessary properties of the histidine it replaces. In this paper, we show that it is possible to activate a chemically modified αHL by photochemical removal of a properly placed protecting group (Fig. 1). Therefore, three different types of stimuli that can activate a pore-forming protein (biochemical, chemical and physical) are now at our disposal.

Caged reagents in biology

Biological reagents that are released by ultraviolet or visible irradiation are often known as 'caged' compounds [29,30]. Considerable effort has been spent on making small caged molecules beginning with the pioneering studies of J.H. Kaplan and colleagues [31] on caged ATP. The vast array of caged molecules now include neurotransmitters (e.g., glutamate) and second messengers (e.g., cAMP and Ca^{2+}). As noted by several authors [29,32], less effort has been spent on the rational design of caged peptides and proteins. There are notable exceptions, for example caged enzymes made with active-site directed reagents [33] or by the introduction of non-natural amino acids during *in vitro* translation [34]. Here, we introduce 2-bromo-2-(2-nitrophenyl)acetic acid (BNPA), a versatile, water-soluble reagent for the protection of cysteine-containing peptides and proteins that is based on the 2-nitrobenzyl chemistry commonly used in caging reagents [29,30]. We show that an active, single-cysteine mutant of αHL is inactivated by reaction with BNPA. When the α-carboxy-2-nitrobenzyl (CNB) group, which is introduced by BNPA treatment, is removed by photolysis, pore-forming activity is regenerated. Caged pore-forming proteins should be useful for the permeabilization of selected cells in a collection of cells and for the permeabilization of a predetermined region of the surface of a single cell.

Results

BNPA: a water-soluble reagent for 'caging' sulfhydryls

The photoremovable 2-nitrobenzyl group has been used for the protection of many functionalities in synthetic organic chemistry [35] and for the production of caged reagents with applications in biology [29]. Relevant to the present study, the sulfhydryl group in cysteine has been protected with 2-nitrobenzyl chloride as S-2-nitrobenzylcysteine for applications in peptide synthesis [36]. Cysteine-containing peptides were generated from protected derivatives in good yields by irradiation at ≥ 350 nm, provided that reagents were present to trap the photoproduct 2-nitrosobenzaldehyde and to prevent sulfhydryl oxidation. In attempts to protect preformed cysteine-containing peptides and proteins, we found that 2-nitrobenzyl chloride and related commercial reagents were too insoluble in aqueous buffers to permit

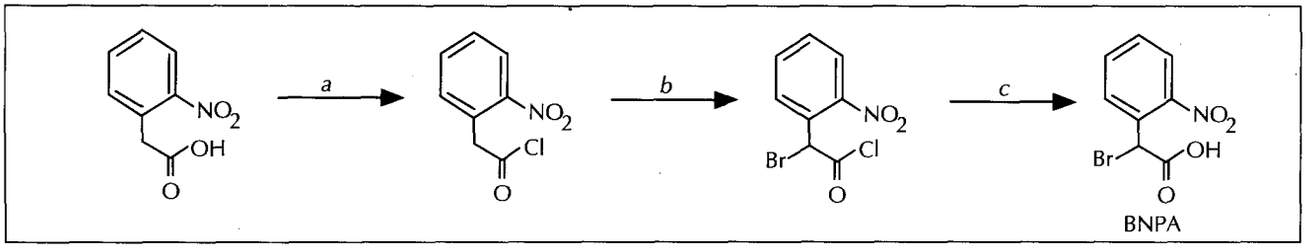

Fig. 2. One-pot synthesis of BNPA. Conditions were as follows: a, $SOCl_2$ in CCl_4, 65 °C, 1.5 h; b, *N*-bromosuccinimide/HBr, 70 °C, 4.5 h; c, ice water, 1 h.

efficient derivatization under most circumstances. Therefore, we made 2-bromo-2-(2-nitrophenyl)acetic acid (BNPA) with which to introduce the α-carboxy-2-nitrobenzyl (CNB) protecting group. The molecule can be produced in high yield by the bromination of 2-nitrophenylacetyl chloride, followed by hydrolysis of the acyl chloride group (Fig. 2). BNPA is highly water-soluble at pH values around neutral, and the α-carboxyl group increases the reactivity of the electrophilic center towards the cysteinate anion. Further, the presumed photoproduct, 2-nitrosoglyoxylic acid (Fig. 3), is less reactive than the 2-nitrosobenzaldehyde photoproduct. In addition, small peptides containing the CNB group should be more soluble in water than those protected with the simple 2-nitrobenzyl group.

Photodeprotection of a model tripeptide: CNB-glutathione

To test the potential of BNPA as a protecting agent for cysteines in peptides and proteins, we reacted the tripeptide glutathione (GSH, γ-Glu-Cys-Gly) with BNPA to form GS-CNB (γ-Glu-CysCNB-Gly). Purified GS-CNB was characterized by UV absorption spectroscopy and amino-acid analysis [37]. When the protected peptide was irradiated at 300 nm in the presence of the reducing agent dithiothreitol (DTT; 5 mM), GSH was released as determined by thin-layer chromatography (TLC), amino-acid analysis and by reaction with 5,5'-dithio(2-nitrobenzoic acid) (Ellman's reagent), which is used to quantitate sulfhydryl groups [38]. The photochemistry of 2-nitrobenzyl derivatives is highly dependent upon pH [39]. Accordingly, we found a higher yield of GSH (~50 %) when irradiation was carried out for the optimal time (30 min, see Materials and methods) at pH 6.0, than at pH 8.5 (~30 %). Oxidation products of GSH, GSSG (oxidized GSH) and GSO_3H (glutathionesulfonic acid), were not detected after 30 min irradiation.

Photodeprotection of a model polypeptide: αHL-S3C

We wished to find out whether BNPA could be used to selectively derivatize a polypeptide containing a single cysteine residue and whether the modified polypeptide could be deprotected by near UV light. We had previously used gel-shift electrophoresis to determine sulfhydryl accessibility in single-cysteine mutants of αHL. Radiolabeled αHL polypeptides were modified with the dianionic 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonate (IASD) and subjected to extended SDS-PAGE. Clean gel shifts to decreased mobility were seen for all cysteine mutants examined. The phenomenon is familiar to researchers who work on protein kinases, as phosphorylation of a polypeptide on a single residue often produces a decrease in mobility upon SDS-PAGE; an increase in mobility would be predicted if the additional negative charge were the only factor involved. No gel shift was observed with wild-type (WT) αHL, which contains no cysteine, and stepwise double shifts were seen for polypeptides containing two cysteines [40]. Therefore, SDS-PAGE of chemically modified αHL polypeptides is diagnostic for the availability of an unmodified cysteine residue, although the physical basis of the gel shifts is obscure. αHL-S3C undergoes a particularly large reduction in electrophoretic mobility upon modification with IASD. Therefore, we used αHL-S3C as a model for studying polypeptide modification with BNPA.

Fortunately, reaction of radiolabeled αHL-S3C with BNPA also produces a substantial gel shift upon prolonged electrophoresis (Fig. 4, lane 3). Because it is less pronounced, it is distinguishable from the shift produced by IASD (Fig. 4, lane 2). By using the gel shift as a criterion, complete modification of S3C was achieved with 20 mM BNPA at pH 8.5 after 1 h at room temperature. Excess reagent was removed by repeated dilution and ultrafiltration. The modified hemolysin, αHL-S3C-CNB was then

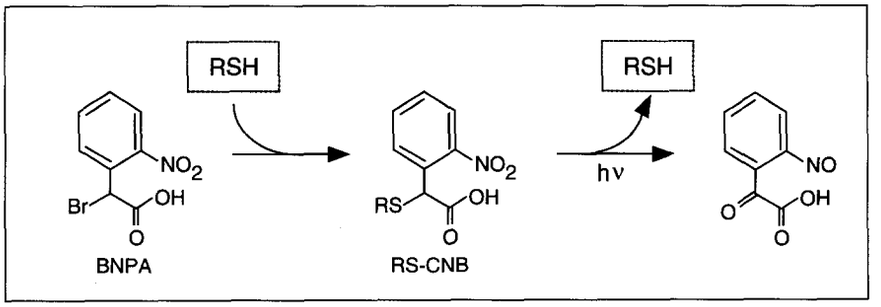

Fig. 3. Formation of CNB-thioethers and their proposed photochemistry. Cysteine-containing peptides and proteins can be selectively modified with BNPA in aqueous buffers at pH 8.5. The CNB group is removed by irradiation at wavelengths (≥ 300 nm) that do little damage to most biological samples. R denotes a peptide or protein.

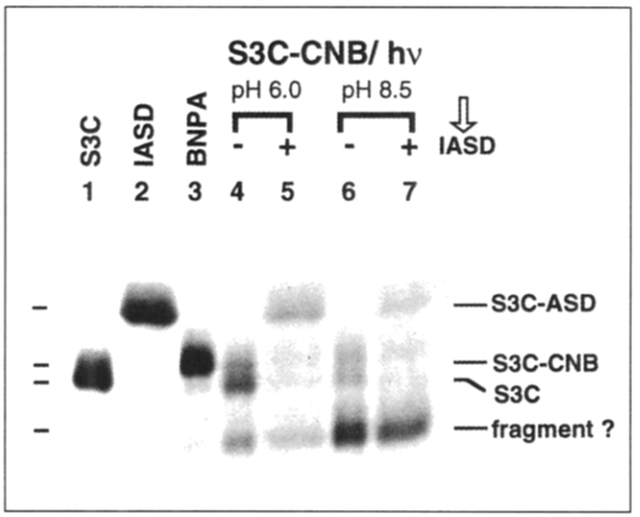

Fig. 4. Chemical modification of αHL-S3C examined by SDS-PAGE. Modifications by the charged reagents result in characteristic gel shifts after extended electrophoresis. ^{35}S-labeled S3C produced by *in vitro* transcription and translation was treated as described in the text and subjected to electrophoresis in a 12 % SDS polyacrylamide gel. An autoradiogram of a region between the 29.5 kD and 45.5 kD markers is shown. Lane 1, S3C; lane 2, S3C treated with IASD; lane 3, S3C treated with BNPA; lane 4, BNPA adduct of S3C photolyzed at pH 6.0; lane 5, as lane 4 but the photolysis products were further treated with IASD; lane 6, BNPA adduct of S3C photolyzed at pH 8.5; lane 7, as lane 6 but the photolysis products were further treated with IASD. Key: S3C, αHL-S3C; S3C-CNB, αHL-S3C adduct formed by reaction of Cys-3 with BNPA; S3C-ASD, αHL-S3C adduct formed by reaction of Cys-3 with IASD.

irradiated at pH 6.0 or pH 8.5 under conditions that had been established to give optimal release of GSH from GS-CNB. At pH 6.0, ~60 % of the αHL-S3C-CNB was converted to a species that comigrated with αHL-S3C upon SDS-PAGE (Fig. 4, lane 4). The new polypeptide contained a free sulfhydryl group as shown by reaction with IASD, which produced a large gel shift (Fig. 4, lane 5) of the same magnitude seen when *bona fide* αHL-S3C is treated in the same manner (lane 2). When irradiation was performed at pH 8.5, far less αHL-S3C-CNB was converted to αHL-S3C and a product of higher electrophoretic mobility was predominant (Fig. 4, lane 6) that did not react with IASD (lane 7). This product presumably arises from fragmentation of the polypeptide chain, although other explanations are possible, such as intramolecular crosslinking or an unanticipated reaction of the CNB group that produces a large shift towards the anode. These points require further investigation.

αHL-R104C-CNB: a caged pore-forming protein

Encouraged by the successful experiments with glutathione and αHL-S3C, we searched for a single-cysteine mutant of αHL that would be inactivated by BNPA modification but reactivated upon irradiation. As an initial screen, we considered active single-cysteine mutants of αHL that are inactivated by covalent modification with IASD. From 83 cysteine mutants in our collection at the time, 22 were candidates by this criterion (B.W., unpublished data). We eliminated four in which binding to the cell surface was affected by modification because, for most applications, it is important that the inactive modified protein is on the surface of the target cell. Five mutants with cysteines in the central loop were also eliminated because we knew that, although they were inactivated by IASD, they were not inactivated by Ellman's reagent, which has structural features resembling the CNB group. Of the remaining thirteen, nine were disregarded in this study because cysteine substitution lowered the hemolytic activity of the polypeptide slightly compared to WT-αHL or because the IASD-modified protein showed a little residual activity. Of the remaining four (R104C, E111C, K168C and D183C), we focus here on αHL-R104C. Residue 104 is in a crucial region of the αHL polypeptide that both flanks the central loop and interacts with residues near the amino terminus that cooperate with the loop in the final step of pore formation (B.W. and R.G. Panchal, unpublished data).

Unmodified αHL-R104C is as active as WT-αHL and, upon modification with IASD, gives an especially pronounced gel shift and has almost no residual activity (B.W., unpublished data). Treatment with BNPA also blocked the pore-forming activity (see below). When the reaction of αHL-R104C with BNPA and subsequent deprotection with near UV irradiation was examined by SDS-PAGE, important differences were seen compared to αHL-S3C. Reaction with BNPA produced the expected decrease in electrophoretic mobility (Fig. 5, lane 3). However, at most, only 80 % conversion to αHL-R104C-CNB was obtained. At first glance, it appeared that unmodified αHL-R104C remained after the reaction. Close examination of the gels and treatment of the BNPA reaction products with IASD (Fig. 5, lane 4), however, revealed the existence of two side-reaction products that migrated close to αHL-R104C, but contained no modifiable sulfhydryl groups. The same result was obtained with highly purified, unlabeled αHL-R104C obtained by expression in *S. aureus* (C.-y. C., C. Shustak and S. Cheley, unpublished data). As these reaction products appeared to be devoid of hemolytic activity, we could proceed to examine the photoactivation of BNPA-modified αHL-R104C.

BNPA-modified αHL-R104C was irradiated at 300 nm at pH 6.0 or pH 8.5. In contrast to the case of αHL-S3C, more αHL-R104C was generated at pH 8.5 than at pH 6.0 (Fig. 5, lanes 6 and 8), and presumed chain cleavage, in this case to a product with M_r ~30 000 (not shown), was far less prevalent. The polypeptide presumed to be deprotected αHL-R104C was shifted to reduced mobility after treatment with IASD, demonstrating the presence of a free sulfhydryl group (Fig. 5, lanes 9 and 10). The large shift with IASD is highly characteristic of *bona fide* αHL-R104C (lane 2). The two unidentified BNPA-modification products were unaffected by irradiation or subsequent treatment with IASD. Based on the amount of material that reacted with IASD, the yield of

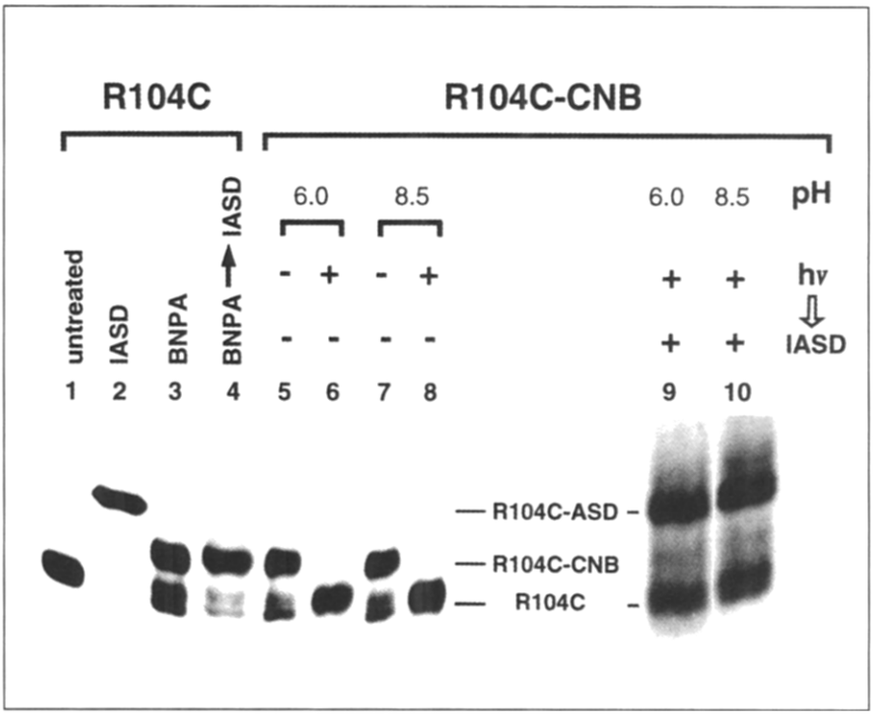

Fig. 5. Chemical modification of αHL-R104C examined by SDS-PAGE. ^{35}S-labeled R104C was treated as described in the text and subjected to extended electrophoresis in a 12 % SDS polyacrylamide gel. An autoradiogram of a region between the 29.5 kD and 45.5 kD markers is shown. Lane 1, R104C; lane 2, R104C treated with IASD; lane 3, R104C treated with BNPA; lane 4, R104C treated with BNPA and then further treated with IASD; lane 5, BNPA adduct of R104C after removal of excess reagent; lane 6, as lane 5 but photolyzed at pH 6.0; lane 7, BNPA adduct of R104C after removal of excess reagent; lane 8, as lane 7 but photolyzed at pH 8.5; lane 9, as lane 6 but the photolysis products were further treated with IASD; lane 10, as lane 8 but the photolysis products were further treated with IASD. Key: R104C, αHL-R104C; R104C-CNB, R104C adduct formed by reaction of Cys-104 with BNPA; R104C-ASD, R104C adduct formed by reaction of Cys-104 with IASD. The presumed fragmentation product is not in the displayed section of the gel.

αHL-R104C was ~60 %. The actual yield of αHL-R104C from αHL-R104C-CNB is somewhat higher, considering that not all of the αHL-R104C could be derivatized with BNPA. The pH-dependence for optimal deprotection versus chain cleavage is the opposite to that seen with αHL-S3C and suggests that partitioning between the two reaction products depends on the environment provided by the folded polypeptide chain. This point requires further investigation.

Photodeprotection of αHL-R104C-CNB yields a functionally active hemolysin

To determine whether the photoreleased αHL-R104C had pore-forming activity, hemolysis assays were performed with rRBC. WT-αHL that had been treated with BNPA was included as a control to determine whether UV irradiation decreased the activity of αHL. In a second control, αHL-H35C-CNB was included to demonstrate that photoproducts generated from residual components of the *in vitro* protein synthesis and chemical modification procedures do not cause rRBC lysis. H35C is an inactive single-cysteine mutant of αHL [28,40].

WT-αHL showed strong lytic activity before irradiation (Fig. 6a, open circles), while αHL-H35C-CNB and αHL-R104C-CNB were inactive (Fig. 6b,c, open circles). After irradiation at 300 nm, WT-αHL retained its activity, whereas irradiated αHL-H35C-CNB remained inactive (Fig. 6b, filled circles). Gratifyingly, αHL-R104C-CNB was activated by irradiation (Fig. 6c, filled circles). The extent of reactivation, determined by a quantitative microtiter assay, was 10–15 % of the value expected if complete conversion to αHL-R104C had occurred. In practice, conversion was ~60 %, as ascertained by SDS-PAGE (Fig. 5). The apparent lack of agreement with the microtiter assay is addressed in the Discussion. By using αHL-R104C-CNB made from unlabeled αHL-R104C obtained by expression in *S. aureus* (C.-y. C., C. Shustak and S. Cheley, unpublished data), which was available at higher concentrations and in high purity, appreciable lysis could be observed a few minutes after photoactivation (Fig. 6d). In these experiments, αHL-R104C-CNB was activated in solution and then added to rRBC. Given the prospective applications in cell biology, it is important to note that lysis was also induced when αHL-R104C-CNB was activated on intact rRBC (data not shown).

Discussion

Photogenerated pore-forming proteins (photopores)

Photoactivatable 'caged' molecules are used for generating reagents on or in cells at defined times and in defined regions [29,30]. The caging and release of small molecules or ions, such as cAMP and Ca^{2+}, has been the subject of numerous studies, but relatively little work has been done on peptides and proteins. Here, we describe a one-pot synthesis of a water-soluble cysteine-directed reagent, BNPA, for caging peptides and proteins with the CNB protecting group, which has been used previously for caging small molecules [41–43]. With BNPA, we have produced a caged pore-forming protein by protecting a mutant of staphylococcal α-hemolysin containing a single cysteine, αHL-R104C, to form αHL-R104C-CNB. Upon irradiation with near UV light, this 'photopore' is activated and permeabilizes cell membranes, as demonstrated by assaying the lysis of rRBC.

We had previously observed the ability of inactive mutants of αHL to inhibit the activity of WT-αHL [16,20]. Such behavior, an *in vitro* dominant negative effect, was not

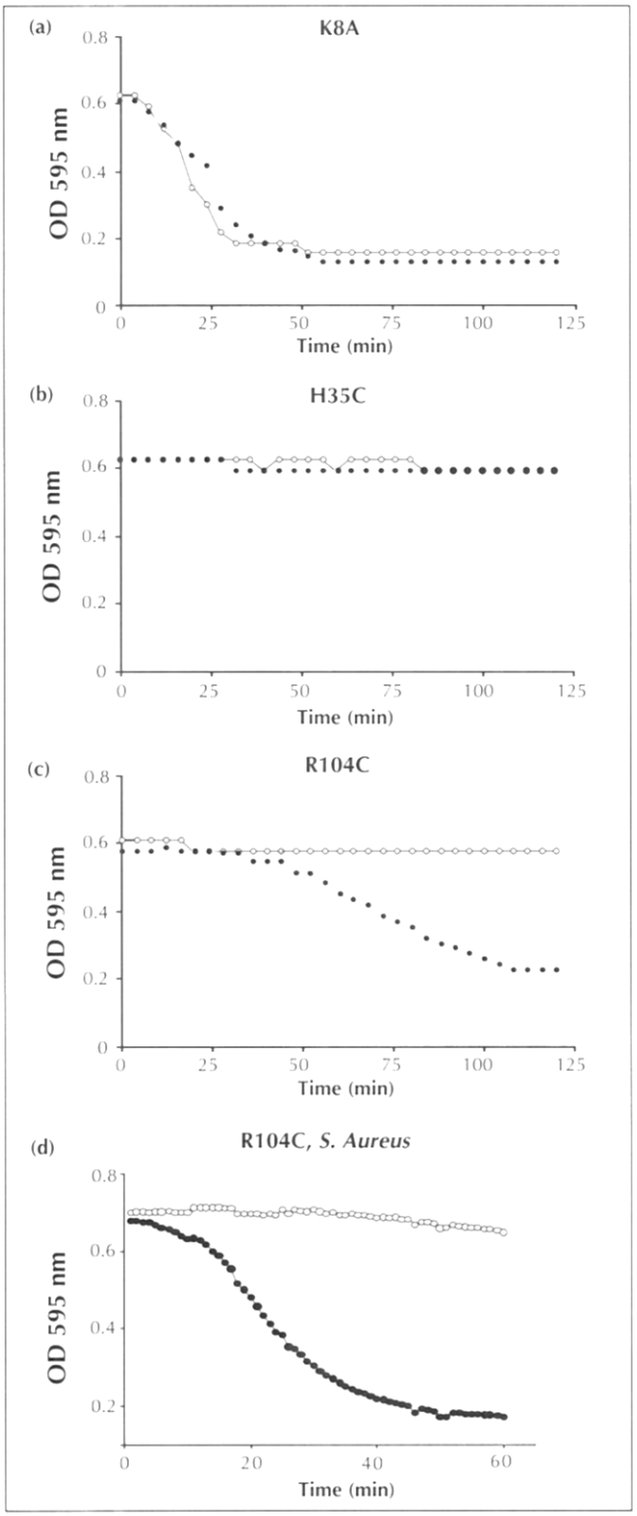

Fig. 6. Activation of αHL-R104C-CNB by irradiation at 300 nm. Hemolysins **(a)** K8A (WT), **(b)** H35C and **(c)** R104C were prepared by *in vitro* transcription and translation and were treated with BNPA. Excess reagent was removed by repeated ultrafiltration. **(d)** Hemolysin R104C, prepared by expression in *S. aureus*, was also modified with BNPA. In this case, excess reagent was removed by gel filtration. The hemolysins were irradiated at 300 nm (●) or not further treated (○). Hemolytic activity towards rRBC was measured by monitoring light scattering at 595 nm. The concentration of each protein in the assay was 0.5 μg ml^{-1}, with the exception of (d) where the concentration was 2.0 μg ml^{-1}.

unexpected for the formation of a multisubunit pore. Therefore, we were concerned that incomplete deprotection of αHL-R104C-CNB, contamination with other products of the chemical modification procedure (see above) or the production of photoproducts other than properly deprotected αHL-R104C might prevent pore formation. In practice, sufficient αHL-R104C is regenerated to cause efficient lysis of rRBC, although the lytic activity (10–15 % of the theoretical maximum) is indeed less than predicted from the release of αHL-R104C (~60 %). An important implication is that, when yields are modest, it is far more practicable to turn on an activity by photodeprotection, as we do here, than it is to turn an activity off. In terms of applying caged peptides and proteins in experimental biology, an increase in activity from a negligible level is useful, but a decrease in activity to, say, 85% of the initial level will not usually be helpful.

The action of photolyzed αHL-R104C-CNB may be too slow (Fig. 6d) for certain experiments such as the production of very rapid local transmembrane fluxes of Ca^{2+}. Removal of the 2-nitrobenzyl group is rapid, however [42,44], and it is very likely that the principles developed here can be extended to related agents that cause more rapid cell permeabilization. Furthermore, our assay monitored the colloid osmotic lysis of rRBC, which has an extended lag phase; the exchange of small molecules across the cell membrane occurs long before lysis [45].

We are also considering methods for switching αHL on and off again, rather than merely triggering its activity. One approach is to combine the photochemical trigger with a previously developed technique for pore closure. For example, it should be possible to make photoactivated pores that are closed by low concentrations of divalent metal ions [19]. Another approach is to use a photoisomerizable group for the targeted chemical modification of a key single cysteine. For example, spiropyrans, azobisbenzenes and related molecules have been used to produce switchable enzymes by random protein modification [46]. In a study relevant to the present work, an attempt was made to produce a photogated dimer from the peptide ionophore gramicidin A by bridging two molecules with an azobisbenzene linker [47]. Although interesting differences in channel characteristics were seen, light-dependent on–off switching was not observed.

Applications of photogenerated pores

Photopores have potential applications in basic science and biotechnology. While RBC undergo colloid osmotic lysis when treated with αHL, most other cells do not [2]. Thus, αHL-R104C-CNB and related caged hemolysins should be useful for cell permeabilization. Permeabilization with pore-forming proteins compares favorably with other techniques for introducing molecules into cells, including microinjection and electroporation. This is especially true when pore-forming proteins that can be triggered or switched on and off are considered.

αHL binds irreversibly to membranes [14] and is unlikely to diffuse from one cell to another. Therefore, selective permeabilization of one cell in a collection should be possible with αHL-R104C-CNB and related reagents by directing light at the target. The question of diffusion of αHL on the surface of a cell remains to be explored. This will define the time interval for which permeabilization can be confined to one part of a single cell, thereby allowing the exchange of reagents into defined regions of the interior. Of course, the effective activity of the pore would be greatly reduced by diffusional dilution and this might provide a convenient way of shutting down activity.

The effective internal diameter of WT αHL is 1–2 nm. Therefore, both macromolecules and organelles are retained by cells permeabilized with this reagent. It is important to note that α-hemolysin is not the only pore-forming protein that might be caged by the procedure we have developed. For example, we are conducting related experiments on streptolysin O, which forms pores of > 30 nm diameter in membranes. With this reagent, organelles are retained while many macromolecules, including IgG molecules [2] and oligonucleotides [48], can pass.

Photopores might also have applications in pharmacology, for example in drug encapsulation [4]. It is conceivable that drugs trapped in liposomes could be released at specific sites in the body by opening pores in the bilayers with light. Caged pore-forming proteins might also be useful for cell or enzyme encapsulation. Encapsulated cells show promise for treating problems such as hormone deficiencies, and encapsulated enzymes may be used to treat diseases of metabolism. In both cases, the encapsulation system could be regulated by controlling access through the opening and closing of pores.

Pore-forming proteins have further potential as components of sensors for various analytes including metal ions [19,49]. Photopore technology might be used to produce addressable two-dimensional arrays of pores with patterned open and closed states.

General utility of caged peptides and proteins

The properties of polypeptides other than pore-forming proteins, such as enzymes and signal transduction molecules, might be extended by caging with BNPA. For example, caged protein kinases or GTP-binding proteins would be helpful in studies of cell signaling. It will be possible to activate caged proteins in a selected region of the cell, such as the nucleus, perhaps by using two-photon irradiation techniques [43]. In biophysical studies, conformational changes of proteins in crystals or protein folding in solution might be rapidly initiated by photolysis.

The technique outlined here is particularly powerful when combined with cysteine-scanning mutagenesis, as we describe for αHL. During this work, we also completed a study of cysteine mutants of αHL in which pore-forming activity was examined before and after modification with the bulky, dianionic reagent IASD. This allowed us to select potential candidate cysteine mutants that might also have been inactivated by the less drastic modification brought about by BNPA treatment. In principle, there is no reason why BNPA could not be used directly on the large sets of cysteine mutants that now exist for several polypeptides. The extent of IASD modification can be readily evaluated by charge-shift electrophoresis [40], but, as we show here, BNPA modification can also produce substantial gel shifts. We have also shown that the extent of modification with BNPA can be determined by a subsequent treatment with IASD.

The possibilities for caged peptides are numerous and include the local release of enzyme inhibitors at specific sites within cells and the unmasking of peptides that block specific protein–protein interactions. BNPA can be used to protect cysteines in synthetic peptides after they have been fully deprotected, as demonstrated here by the modification of glutathione. BNPA might also be used to block cysteines or cysteine analogs that are placed in large synthetic peptides by chemical ligation [50]. Unnatural amino acids with reactive side chains introduced by chemical synthesis, site-specific modification [51] or *in vitro* translation [52] might also be protected with BNPA.

Caged peptides and proteins should also have applications in pharmacology [53]. Biologically active peptides and proteins could be released at specific sites in the body in predetermined doses and at defined times. Two-photon activation might allow highly localized activation with radiation that is more penetrating than near UV.

The procedure might also be extended to non-peptide biopolymers containing sulfhydryl groups and, in the absence of sulfhydryls, it might be possible to selectively modify less reactive nucleophilic groups. The gross physical characteristics of synthetic polymers protected at multiple sites with BNPA would be altered by photochemical deprotection. The loss of charge could be used to modulate properties such as solubility [54], the ability to penetrate biological membranes and ion-exchange capacity.

BNPA is a prototype reagent for caging peptides and proteins. An obvious extension, based on previous work with caged molecules, is to alter the chromophore (while retaining the 2-nitrobenzyl functionality) so that the absorption maximum is pushed into the visible range. Biological samples irradiated at longer wavelengths might suffer less direct and indirect damage, for example sensitized photooxidation. We also recognize that sulfhydryl-directed protecting groups, based on the 2-nitrobenzyl functionality, that are more highly charged or more bulky might be required to inactivate certain target proteins or peptides. Further, the bromide leaving group on BNPA might be replaced with iodide or with even more reactive leaving groups. Several possibilities for photodeprotection

based on chemistries distinct from 2-nitrobenzyl chemistry are also available [29]. The chiral center in BNPA that must yield diastereomeric cysteine adducts could also be a disadvantage in some circumstances and might be remedied without too much difficulty.

Polypeptide chain cleavage by CysCNB

Remarkably, polypeptide chains modified on cysteine with BNPA appear to be cleaved when irradiation is carried out at pH values that are not optimal for deprotection (Fig. 4). This finding could have applications in both basic science and biotechnology. For example, it might be applied to the cleavage of recombinant fusion proteins or the removal of synthetic peptides from solid supports. It could also be used to trigger the inactivation of polypeptides (such as enzymes) by chain cleavage. The details of the chemistry of the presumed chain-cleavage reaction are under investigation.

Significance

Bacterial pore-forming proteins, such as α-hemolysin and streptolysin O, are important reagents that allow the controlled permeabilization of cells. Cell permeabilization has applications in many areas, for example in studies of cell signaling. One drawback to these studies has been the lack of ability to control the activity of the pore-forming protein. It should be possible to overcome this drawback by using photochemistry. Here, we show that it is indeed possible to photoactivate a 'caged' α-hemolysin that has been inactivated by site-directed mutagenesis and targeted chemical modification. The modified α-hemolysin and related molecules will be useful for the permeabilization of one cell in a collection of cells (e.g., a neuron in a network) or one region of a single cell (e.g., the presynaptic terminus of a neuron).

The manipulations described here might also have applications in biotechnology. For example, in nanostructure synthesis, two-dimensional arrays of pores with a pattern of open and closed states are accessible using our methodology. In biotherapeutics, light-activated pores might be used to control drug release from capsules such as liposomes.

The water-soluble reagent we have developed for forming a photoactivated α-hemolysin places an α-carboxy-2-nitrobenzyl (CNB) group on sulfhydryl groups and should be generally useful for producing caged peptides and proteins, such as derivatives of protein kinases and their peptide inhibitors. Caged peptides and proteins should have widespread applications in basic science, including uses such as the triggering of biological reactions for examination by time-resolved biophysical techniques.

Materials and methods

Synthesis of 2-bromo-2-(2-nitrophenyl)acetic acid (BNPA)

The general procedure for α-halogenation of acyl chlorides described by Harpp *et al.* [55] was used. To 2-(2-nitrophenyl)acetic acid (Aldrich, 5.0 g, 27.6 mmol) was added carbon tetrachloride (5 ml) and thionyl chloride (7.95 ml, 109 mmol). The mixture was stirred at 65 °C for 1.5 h to form the acyl chloride, after which *N*-bromosuccinimide (5.90 g, 33.1 mmol), CCl_4 (25 ml) and a catalytic amount (11 drops) of HBr in acetic acid were added to the flask. The mixture was then heated at 70 °C. After 4.5 h, ice (25 g) was added to the cooled mixture, which was stirred vigorously for 1 h to hydrolyze the acyl chloride. The CCl_4 layer was retained and the aqueous phase was extracted with 3 x 25 ml CH_2Cl_2. The combined organic fractions were dried with Na_2SO_4 and the solvent was removed by evaporation furnishing crude 2-bromo-2-(2-nitrophenyl)acetic acid in near quantitative yield as a brown oil. A portion was recrystallized twice from CH_2Cl_2 yielding a buff solid. UV (10 mM NaP_i, pH 8.5): λ_{max} = 265 nm, ϵ 4700. ^1H NMR ($CDCl_3$): δ 5.50 (1H, br, COOH), 6.13 (1H, s), 7.55 (1H, m, aromatic), 7.71 (1H, m, aromatic), 8.02 (2H, m, aromatic). Elemental analysis. Calculated for $C_8H_6BrNO_4$: C, 36.95; H, 2.33; Br, 30.73; N, 5.39; O, 24.61. Found: C, 37.35; H, 2.48; Br, 27.59; N, 5.43; O, 24.98.

Modification of reduced glutathione (GSH) with BNPA

Stock solutions of the reduced form of glutathione (GSH, 100 mM in water) and BNPA (100 mM in 100 mM NaP_i, pH 8.5) were freshly made and used to prepare a reaction mixture containing 20 mM GSH, 20 mM BNPA in 250 mM NaP_i, pH 8.5, which was incubated overnight at room temperature in the dark. TLC (silica gel, system 1: ethanol/water/ammonia 7:2:1) revealed ~80 % conversion of GSH to a new compound GS-CNB (Rf 0.70, ninhydrin positive, 254 nm absorbing). GSH (Rf 0.59, ninhydrin positive) and GSSG (oxidized GSH) almost comigrated in this system, but further analysis by system 3 (see below) revealed that the remainder of the GSH (~20 %) had been oxidized to GSSG. GS-CNB was purified by preparative TLC (system 1) and isolated in 48 % yield based on quantitative amino-acid analysis [36]. UV (10 mM NaP_i, pH 8.5): λ_{max} = 265 nm, ϵ 4 600.

Photolysis of GSH-CNB

GSH-CNB in water (2 mM, 42.5 μl) was buffered by the addition of 500 mM NaP_i (5.0 μl) at either pH 6.0 or pH 8.5, followed by the addition of freshly prepared DTT solution (100 mM in water, 2.5 μl). The mixture was placed on ice in a microtiter plate well and photolyzed at 300 nm, 3.5 cm from a Fotodyne UV illuminator (Foto UV 300). Samples (6 μl) were removed at 0, 15, 30, 60, 90 and 120 min and spotted onto a TLC plate, which was eluted with ethanol/water/ammonia (8:1:1, system 2) or isopropanol/2-butanol/acetic acid/water (5.0:5.0:2.6:3.5, system 3). Standards (Rf system 2, ninhydrin color; Rf system 3, ninhydrin color) were: GSH (0.20, pink; 0.22, red), GS-CNB (0.42, pink; 0.24, red) GSSG (oxidized GSH: 0.12, pink; 0.01, orange) and GSO_3H (glutathionesulfonic acid: 0.30, pink; 0.13, orange). Under these conditions, the optimal release of GSH occured after 30 min irradiation.

Synthesis of α-hemolysins by in vitro *transcription and translation (IVTT)*

In vitro synthesis of ^{35}S-labeled αHL polypeptides was carried out in an *E. coli* S30 extract supplemented with T7 RNA polymerase and rifampicin as described [56], except that 1.0 μg of plasmid DNA was added to a 25 μl reaction. The mutant αHL

genes used here were inserted into the plasmid pT7Sf1A [56] and have been or will be described elsewhere: K8A [20] and S3C [40], H35C [28] and R104C (B.W., unpublished data). R104C was derived from K8A, and S3C and H35C from the unaltered αHL gene. K8A has the properties of native αHL, but is less susceptible to inactivation by adventitious proteases [20] and was used as the WT-αHL control in this work.

BNPA modification of single-cysteine mutants

αHL mutants (30 μl IVTT mix) were reduced for 5 min at room temperature by the addition of 10 mM DTT (15 μl in water), 1.0 M Tris HCl, pH 8.5 (30 μl), and water (45 μl). 100 mM BNPA in 100 mM NaP_i, pH 8.5 (30 μl), was then added and the modification reactions were incubated at room temperature in the dark for 1 h (S3C) or 9 h (K8A (mock reaction), H35C, R104C). At the end of the reaction, excess BNPA and its low mass sulfhydryl adducts (e.g. with DTT) were removed by repeated cycles of dilution with 100 mM Tris HCl (pH 6.0 or 8.5) and concentration by ultrafiltration (Amicon, Microcon-3). The final volume (135 μl) was readjusted to 150 μl with 100 mM Tris HCl containing 10 mM DTT so that the concentration of DTT was 1 mM. The final concentration of BNPA and its low mass sulfhydryl adducts was ≤ 20 μM. The modified proteins were stored at −20 °C before use.

BNPA modification of staphylococcal αHL-R104C

αHL-R104C (0.36 mg ml^{-1}) was dialyzed against 10 mM Tris HCl, pH 8.5, containing 5 mM β-mercaptoethanol. Dialyzed R104C (100 μl), 10 mM DTT in water (20 μl) and 1.0 M Tris HCl, pH 8.5 (40 μl) were incubated for 5 min at room temperature before the addition of 100 mM BNPA in 100 mM NaP_i, pH 8.5 (40 μl). After 3 h at room temperature, 1.0 M DTT (5 μl) was added and excess reagents were removed from the protein by gel filtration on Bio-Gel P-2 (Bio-Rad) eluted with 10 mM Tris HCl, pH 8.5, containing 50 mM NaCl. A fraction was desalted and the buffer exchanged for 100 mM Tris HCl, pH 8.5, 1.0 mM DTT by ultrafiltration (Amicon, Microcon-3).

Photolysis of CNB-αHL (caged α-hemolysins)

CNB-αHL in 100 mM Tris HCl (pH 6.0 or 8.5) containing 1 mM DTT (60 μl) was placed in a well of a 96-well microtiter plate and irradiated for 30 min on ice through a 285 nm cut-off filter (Oriel, #51220) at 3.5 cm from the Foto UV 300 illuminator. To assay for the unmasking of the protected cysteine residue, samples of the irradiated αHL polypeptides were treated with IASD and analyzed by SDS-PAGE. For the pH 8.5 sample, irradiated αHL (5 μl) was diluted with 100 mM Tris HCl, pH 8.5 (3 μl), and reacted with 100 mM IASD in water (2 μl) for 1 h at room temperature. For the pH 6.0 sample, irradiated αHL (5 μl) was diluted with 1.0 M Tris HCl, pH 8.5 (2 μl), and water (1 μl) and treated in the same way.

Hemolysis assays

The lytic activity of the αHL polypeptides towards rRBC was measured in microtiter plates as described [16]. Unirradiated or irradiated CNB-αHL in 100 mM Tris HCl, pH 8.5, containing 1 mM DTT (50 μl) was placed in well 1 of a 96-well flat-bottom plate and diluted with 20 mM KP_i, pH 7.4, 150 mM NaCl and 1 mg ml^{-1} BSA (K-PBSA) (50 μl). Twofold serial dilutions were carried out with the same buffer. Washed rRBC were then added to 0.5 % and the plate was incubated at 22 °C for 3 h. Hemolysis kinetics were recorded with a microplate reader (Bio-Rad, Model 3550-UV).

Gel electrophoresis

SDS-PAGE was carried out according to Laemmli [57] in 40-cm long 12 % polyacrylamide gels run at constant 200 V for 40 h. Gels fixed in methanol/water/acetic acid (3:6:1) were dried and subjected to autoradiography or phosphorimager analysis. Prestained markers were from Gibco BRL.

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