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Free Radical Biology & Medicine 41 (2006) 562–567
www.elsevier.com/locate/freeradbiomed

Original Contribution

An ascorbate-dependent artifact that interferes with the interpretation of the biotin switch assay

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Received 11 August 2005; revised 11 February 2006; accepted 2 March 2006
Available online 29 March 2006

Abstract

As an example of an important redox-based protein posttranslational modification, protein *S*-nitrosation of specific cysteines is attracting more and more attention. The methods of detecting protein *S*-nitrosation in vitro or in vivo have been widely used in recent research, especially the biotin switch assay. An increase in band intensity in the presence of ascorbate is thought to be diagnostic for the presence of *S*-nitrosothiols. However, we found that this is a flawed assumption. In this study, bovine serum albumin (BSA) and even BSA prereduced by 20 mM 2-mercaptoethanol give false-positive signals for *S*-nitrosothiols (corresponding to a level of about 0.5–1% *S*-nitrosated BSA) when detected by the biotin switch assay. Higher blocking conditions could not diminish the signal, whereas omitting ascorbate in the step before biotinylation resulted in the disappearance of the signal. Further investigation of the mechanism showed that ascorbate increases the rate of the biotinylation reaction and accelerates the presence of the false-positive signal. Our results provide direct evidence that ascorbate could give rise to a significant false-positive signal in the biotin switch assay. Ascorbate treatment can interfere with the interpretation of the data. Hence, care should be taken when this method is used.

Keywords: *S*-Nitrosation; Ascorbate (ascorbic acid); Biotin switch assay, Free radical

Introduction

S-Nitrosation, which involves the formation of an *S*-nitroso function group on a protein cysteine residue, has emerged as an important mechanism for dynamic, posttranslational regulation of most or all main classes of protein. *S*-*N*itrosation thereby conveys a large part of the ubiquitous influence of nitric oxide (NO) on cellular signal transduction, and provides a mechanism for redox-based physiological regulation [1]. Initial studies relied to a great extent on in vitro analysis using exogenous NO sources, which did not necessarily recapitulate the cellular milieu. However, a substantial body of recent work has directly implicated *S*-nitrosation in the regulation of numerous signaling pathways in intact cellular systems, for example, the *S*-nitrosation of parkin in the regulation of the E3 ubiquitin ligase activity in Parkinson's disease [2,3]. Therefore, the availability of sensitive methods to detect protein *S*-nitrosation is becoming increasingly important.

At present, several methods are used to detect protein *S*-nitrosation: the Saville-Griess assay [4], the chemiluminescence assay [5,6], and the biotin switch assay [7,8]. The basic principle of the biotin switch assay is that nitrosated cysteines are converted to biotinylated cysteines. The biotin switch assay is suitable for further protein identification and is also valuable for proteomic approaches [9,10]. Therefore, the biotin switch assay is becoming the most widely used method in the detection of protein *S*-nitrosation.

Some researchers have used this assay to analyze endogenous protein *S*-nitrosothiols [3,7,11–13]. Recently, Zhang et al. reported that the biotin switch assay is only

Abbreviations: BSA, bovine serum albumin; biotin–HPDP, *N*-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamide; biotin-M, biotin-maleimide; GSNO, *S*-nitrosoglutathione; MMTS, methylmethanethiosulfonate; MPB, 3-(*N*-maleimidopropionyl)-biocytin; NEM, *N*-ethylmaleimide; NO, nitric oxide; PrS-NO, protein *S*-nitrosothiols; 2-ME, 2-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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doi:10.1016/j.freeradbiomed.2006.03.006

suitable for the detection of protein *S*-nitrosothiols at levels of nanomole (nmol) per milligram (mg) of protein, which is two or three orders of magnitude higher than the endogenous level of protein *S*-nitrosothiols [14]. In their study, they assessed the ability of ascorbate to reduce *S*-nitrosothiols and showed that ascorbate is a very inefficient reducing agent and that higher concentrations of ascorbate and longer incubation times can significantly improve immunological detection of *S*-nitrosothiols.

In this study we found that bovine serum albumin (BSA) and even BSA prereduced by 20 mM 2-mercaptoethanol (2-ME) give false-positive signals for *S*-nitrosothiols when detected by the biotin switch assay, in which ascorbate plays a crucial role. The mechanism was studied and the appropriate use of ascorbate in the biotin switch assay is discussed.

Materials and methods

Chemicals

*S-N*itrosoglutathione (GSNO), as a nitrosating agent, was synthesized from glutathione using acidified nitrite. Glutathione (GSH), ascorbate (ascorbic acid), *N*-ethylmaleimide (NEM), 3-(*N*-maleimidopropionyl)-biocytin (MPB), biotin-maleimide (biotin-M), streptavidin-horseradish peroxidase (streptavidin-HRP), neocuproine, and lysozyme from chicken egg white were purchased from Sigma (St. Louis, MO). *N*-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin–HPDP) and methylmethanethiosulfonate (MMTS) were purchased from Pierce. Different sources of bovine serum albumin fraction V (BSA) were from Geneview, Boehringer Mannheim, or Sigma. All the other chemicals were local products of analytical grade.

Preparation of S-nitrosated proteins

BSA (0.5 or 5 mg/ml) was incubated with GSNO (20 or 400 μM, respectively) at room temperature for 1 h and then GSNO was removed by the addition of 2 vol of –20°C acetone followed by centrifugation at 12,000 *g* for 10 min. The protein pellet was then resuspended in 200 μl HEN buffer. The precipitation step was repeated three times to remove the excess GSNO. Lysozyme (which has no free thiols) was used in place of BSA as a control.

Detection of protein S-nitrosothiols by the Saville-Griess assay

The amount of protein *S*-nitrosothiols (PrS-NO) incorporated into the BSA was determined using the Saville-Griess assay as described [4]. The same experiment was conducted with lysozyme as a negative control. The samples were incubated with 1% sulfanilamide, 0.5 M HCl with or without 0.02% $HgCl_2$, and 200 μl 0.1% naphthalenediamine for 30 min. The samples were then analyzed at 540 nm using a microplate spectrophotometer. The concentration of protein *S*-nitrosothiols was quantified according to the standard curve of sodium nitrite ($NaNO_2$).

Detection of protein S-nitrosothiols by biotin switch assay

In the first step of this assay, free thiols are blocked by incubation with the thiol-specific methylthiolating agent methylmethanethiosulfonate. After the blocking of free thiols, nitrosothiol bonds are selectively decomposed with ascorbate, which results in the reduction of nitrosothiols to thiols. In the last step, the newly formed thiols are reacted with *N*-[(6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP), a sulfhydryl-specific biotinylating reagent. Labeled proteins can easily be detected by immunoblotting with antibodies against biotin (anti-biotin), following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or purified using immobilized streptavidin, a biotin-binding protein [7,8]. Briefly, samples were suspended in 40 μl blocking buffer with MMTS (2.5% (w/v) SDS and 20 mM MMTS in HEN buffer). The free thiols were blocked at 50°C for 25 min with frequent vortexing. The excess MMTS was removed by ice-cold acetone precipitation for 20 min and centrifugation at 12,000 *g* for 10 min. The pellet was recovered in 40 μl HENS buffer (1% (w/v) SDS in HEN buffer) with 0.2 mM biotin-HPDP and 10 mM ascorbate and incubated for 1 h at room temperature. After adding 2× SDS-PAGE loading buffer (nonreducing), 5.0 μg protein was loaded onto the gel and analyzed by SDS-PAGE in the dark and the biotinylated proteins were detected by Western blotting using the streptavidin-HRP (1:10,000) and enhanced chemiluminescent detection (Super-Signal West Pico Trial Kit, Pierce).

In some experiments, the concentration of MMTS was varied or was replaced by NEM. The concentration of ascorbate was varied from 1 to 10 mM, as indicated.

Statistical analysis

The result shown is a representative of at least three independent experiments. Data with $p < 0.05$ were considered significant.

Results and discussion

The detection of protein S-nitrosothiols using the Saville-Griess assay

As shown in Fig. 1, in the first group of samples, where 0.5 mg/ml BSA was incubated with 20 μM GSNO, there is no significant difference compared with lysozyme, which means that the level of PrS-NO generated was lower than the detection limit of the Saville-Griess assay. In the second group, where 5 mg/ml BSA was incubated with 400 μM GSNO, about 3.8 μmol nitrosothiol was generated in the 10-times diluted sample; i.e.; there was 38 μmol nitrosothiol generated in total and the percentage of *S*-nitrosated BSA to total BSA (5 mg/ml, 77 μM) was about 50%.

There are four disulfide bonds and no free thiols in lysozyme; thus it cannot be *S*-nitrosated by GSNO and so was used as a negative control. The results of the Saville-Griess assay confirmed that no significant degree of *S*-nitrosation occurred in lysozyme even in the high concentration

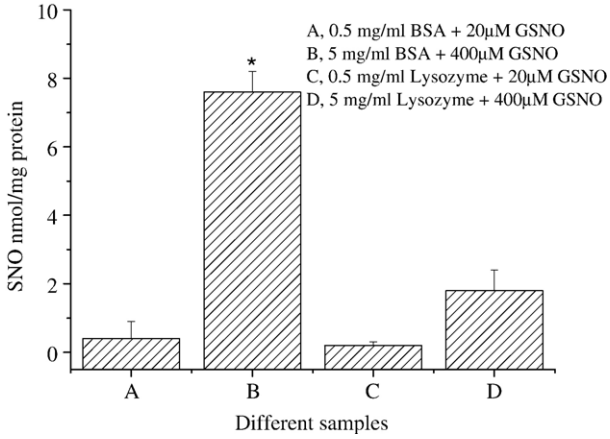

Fig. 1. Protein *S*-nitrosothiols (PrS-NO) detected by Saville-Griess assay. BSA (400 μl) or lysozyme was treated with GSNO for 1 h at room temperature and the excess GSNO was removed by precipitation three times with ice-cold acetone. BSA (5 mg/ml) was diluted (to 0.5 mg/ml) before carrying out the Saville-Griess assay. Data are the means ± SE, $n = 3$; * significant difference between BSA and lysozyme, $p < 0.05$.

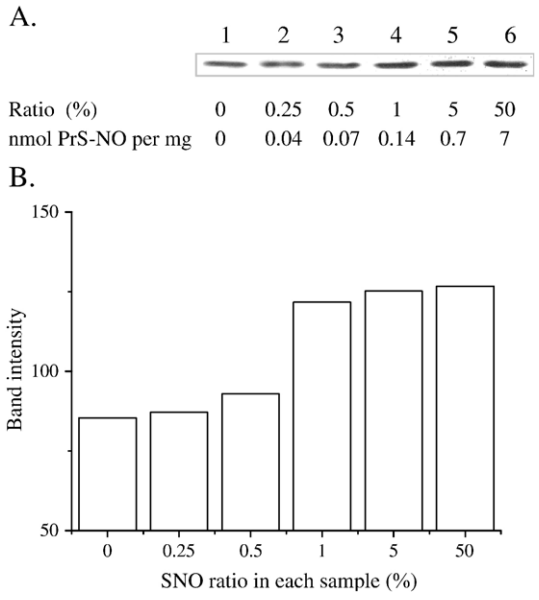

Fig. 2. Protein *S*-nitrosothiols (PrS-NO) detected by the biotin switch assay. (A) The percentage of *S*-nitrosated BSA varied from 0.25, 0.5, 1, 5, to 50%. The concentrations of PrS-NO were calculated as nanomole PrS-NO per milligram protein. There are significant artificial signals in the negative control (band 1) that can hardly be distinguished from the samples with 0.25 and 0.5% *S*-nitrosated BSA (bands 2 and 3). (B) The densitometric analysis of the above results.

treatment group. The trace of nitrosothiol in lysozyme was due to residual GSNO remaining after the precipitation procedure.

The biotin switch assay detects a significant nitrosothiol signal in non-GSNO-treated BSA

Different ratios of *S*-nitrosated BSA from Fig. 1, which contains about 1 SNO per 2 BSA or approximately 7 nmol/mg protein, were taken and diluted with untreated BSA to give SNO levels of 0, 0.25, 0.5% 1, 5, and 50%, corresponding to 0, 0.04, 0.07, 0.14, 0.7, and 7 nmol SNO/mg protein, respectively. The SNO levels were analyzed using the biotin switch assay as shown in Fig. 2. The amount of *S*-nitrosation in the 1, 5, and 50% groups (bands 4, 5, and 6) is significantly higher than the other groups (0, 0.25, and 0.5%). However, the difference between the control group (0% group, band 1) and the positive groups (0.25 and 0.5% group, bands 2 and 3) cannot be distinguished by the biotin switch assay. In other words, when the SNO ratio is lower than 1% in our experiment (0.14 nmol PrS-NO per mg protein), it is hardly distinguished from the control group (0% SNO). In this case, our results are consistent with the results of Zhang et al. [14], who has pointed out that total cell protein can only be observed by Biotin Switch at concentrations in the nanomole per milligram protein range; for a single isolated protein, the sensitivity is about 100 pmol/mg protein. The question is: Where does the obvious nitrosothiol signal in the negative control group come from?

Higher blocking conditions could not diminish the signal in non-GSNO-treated BSA

The biotin switch *S*-nitrosation assay is based on the assumption that only the *S*-nitrosated cysteine residue will be biotinylated. However, if the free thiols of the cysteine residues have not all been successfully blocked with the methylthiolating reagent MMTS, then the nonblocked cysteines may produce a signal in this assay. We first speculated that the signal in the negative control was from the incomplete blocking of the free thiols in BSA. Therefore, we increased the concentration of MMTS from 20 to 50 mM. However, the results showed that the signal did not change any further (Fig. 3). We used another free thiol blocking agent NEM which, unlike MMTS, is irreversible. However, the signal was still not reduced. We then thought that endogenous biotinylated protein could be a source of significant

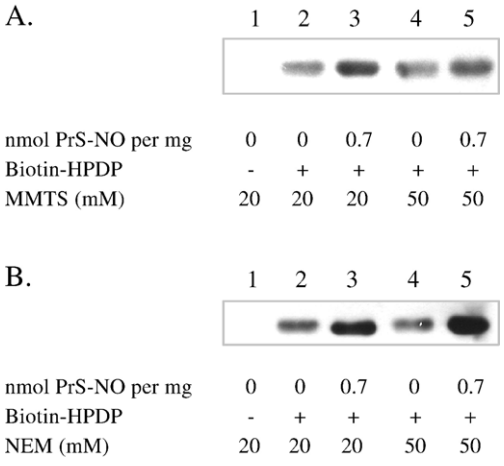

Fig. 3. The effect of higher blocking conditions on the signal in non-GSNO-treated BSA. Increase in the concentrations of the reversible blocking agent MMTS (A) or irreversible blocking agent NEM (B) does not decrease the signal in the negative control (band 2 and band 4).

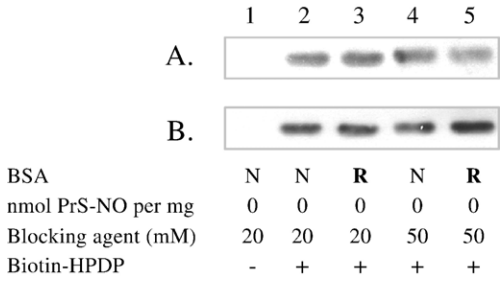

Fig. 4. Detection of the nitrosothiol signal in reduced BSA. BSA was pretreated with the reducing agent 2-ME (20 mM) and then analyzed by biotin switch assay. R, reduced BSA; N, normal BSA. (A) and (B) represent the treatment with different blocking agents MMTS and NEM, respectively.

background because this assay includes biotin immunoblotting. However, this possibility was ruled out by the observation that there was no nitrosothiol signal in the biotin-HPDP negative group (Fig. 3, band 1).

The same signal was observed for reduced BSA

In human blood, albumin is the predominant *S*-nitrosated protein with a level of about 30 nM [15]. One could speculate that since commercial BSA was mostly obtained by ethanol precipitation, the endogenous *S*-nitrosation of BSA in serum might be preserved. There is one free thiol at Cys-34 on the surface of BSA which could be *S*-nitrosated in the sample. We treated the BSA solution with 20 mM 2-ME for over 24 h to eliminate the potential endogenous *S*-nitrosation and then analyzed the *S*-nitrosation in this reduced BSA. The results were the same as for untreated BSA, which means that the signal does not come from the endogenous *S*-nitrosation of BSA (see Fig. 4).

It is ascorbate that enhances the signal in non–GSNO-treated BSA

We omitted ascorbate in the step before biotinylation by biotin-HPDP and found that the signal in the negative control disappeared whether blocking was carried out using MMTS (Fig. 5A) or NEM (Fig. 5B). Treatment with 10 mM ascorbate

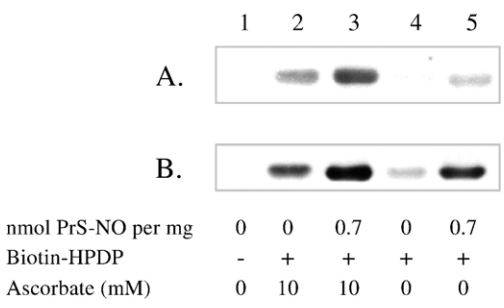

Fig. 5. The detection of nitrosothiols by biotin switch assay with or without ascorbate treatment. Signals in bands 2 and band 3 treated with 10 mM ascorbate during the biotinylation step are significantly higher compared with those in bands 4 and band 5 without ascorbate treatment. (A) Blocked by 20 mM MMTS; (B) blocked by 20 mM NEM.

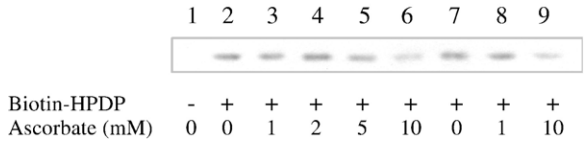

Fig. 6. The effect of ascorbate on the disulfide bonds of lysozyme. Lysozyme, which has 4 disulfide bonds and no free thiols, was treated with biotin-HPDP and different concentrations of ascorbate for 1 h at room temperature, after which the degree of biotinylation was analyzed. The exposure time was lengthened to enhance the signals. The biotinylation signals did not increase with increasing concentration of ascorbate, indicating that no free thiols are produced by reduction of disulfide bonds by ascorbate.

during biotinylation (Fig. 5, bands 2 and 3) gives a significantly higher signal compared to samples without ascorbate treatment (Fig. 5, bands 4 and 5).

Two possible mechanisms

The mechanism of the false-positive result needs further investigation. According to our results using BSA and lysozyme, there were two potential mechanisms to explain the data:

1. Ascorbate could reduce the blocked thiols or the disulfide bonds in proteins and the released free thiols produce the artificial signals. In this case, the signal should increase with increasing concentration of ascorbate.
2. Ascorbate interferes with the process of biotinylation of unblocked free thiols. For the first hypothesis, the reduction process depends on the redox potentials of the reactive substances. The redox potential of cystine/cysteine is about-340 mV and that of dehydroascorbate/asorbic acid is 58 mV [16], which means that the reduction of disulfide bonds by ascorbate is thermodynamically unfavorable. In fact, in our results using lysozyme shown in Fig. 6, increasing the ascorbate concentration does not result in an increase in the artificial signal.

In order to test the second hypothesis above, we analyzed the time course of the biotinylation process and the effects of ascorbate and heat treatment on this process as shown in

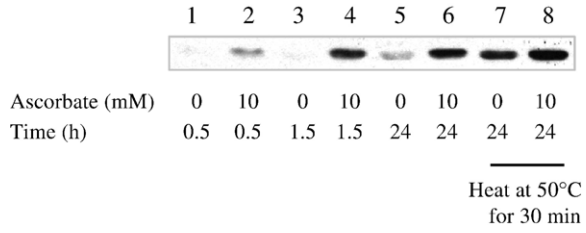

Fig. 7. The time course of the biotinylation process and the effects of ascorbate and heat treatment on this process. Normal BSA was analyzed by biotin switch assay with different incubation times in biotin-HPDP, as indicated. Samples in band 7 and band 8 were incubated at 50°C for 30 min before the normal biotinylation process at room temperature. As the time of biotinylation was increased, the false-positive signal increased significantly (bands 1, 3, and 5). Both ascorbate and heat treatment accelerated the presence of the false-positive signal (bands 2, 4, 6, 7, and 8).

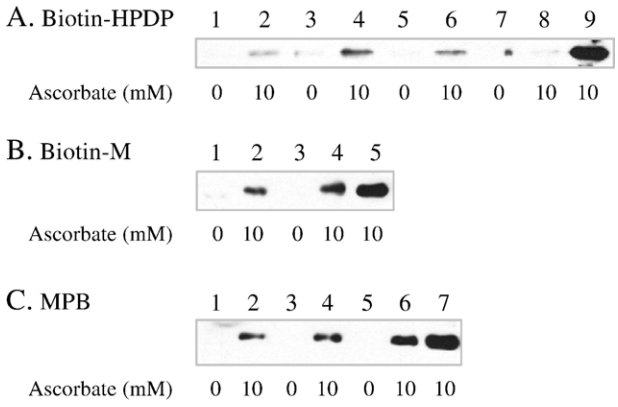

Fig. 8. The same results were achieved with different sources of BSA or different biotinylation reagents. (A) Biotin-HPDP as the biotinylation reagent. Bands 1, 2, 7, 8: BSA from Genview. Bands 3, 4: BSA from Boehringer Mannheim. Bands 5, 6: BSA from Sigma. Bands 1 to 6 were BSA reduced by 20 mM 2-ME for 24 h and then analyzed using the biotin switch assay. Band 9 was normal BSA without MMTS blocking and biotinylated directly by biotin-HPDP. (B) The same analysis using biotin-maleimide (biotin-M). Band 5 was normal BSA without MMTS blocking and biotinylated directly by biotin-M. (C) The same analysis by 3-(*N*-maleimidopropionyl)-biocytin (MPB). Band 7 was normal BSA without MMTS blocking and biotinylated directly using MPB.

Fig. 7. As the time of biotinylation increased, the false-positive signal increased significantly (bands 1, 3, and 5). The false-positive signal in the sample containing ascorbate increased much more significantly, which indicates that ascorbate can accelerate this process. When we incubated the samples at 50°C for 30 min before the normal biotinylation for 24 h, the signal (Fig. 7, band 7) increased significantly compared with the same sample before the heat treatment (Fig. 7, band 5). The fact that heating also speeds up the reaction suggests that biotinylation is an endothermic process. In short, both ascorbate and heat treatment accelerated the presence of the false-positive signal (bands 2, 4, 6, 7, and 8).

It is important to show that this problem is a general one and is not simply an issue with one protein. When we examined different sources of BSA, similar results were obtained (Fig. 8A). In addition, we tried different sources of biotin-HPDP in order to exclude the possibility of contamination, and the same results were also obtained (Fig. 8A). Different biotinylation reagents such as biotin-maleimide (biotin-M) and 3-(*N*-maleimidopropionyl)-biocytin were also tested and the same results were obtained, as shown in Figs. 8B and C.

Our results indicate that treatment with ascorbate can give rise to a significant signal in the biotin switch assay and the level of this false-positive signal is sometimes even higher than the endogenous level of protein *S*-nitrosothiols. A positive sample with less than 1% *S*-nitrosated protein can hardly be distinguished from the negative control because of the presence of the false-positive signal, which may be one reason for the limited sensitivity of the biotin switch assay. In some research papers [17], interpretation of the data are dependent on the assumption that ascorbate does not induce any artificial signals and it only reduces the endogenous PrS-NO to Pr-SH. Taking into account our results, ascorbate may increase the rate of the biotinylation process resulting in limitation of the sensitivity of the biotin switch assay. If one diagnoses the presence of *S*-nitrosothiols (SNOs) only based on the increase in band intensity in the presence of ascorbate, the conclusion would be wrong. In the future, there may be increasing numbers of researchers using this simple and convenient method to detect the degree of endogenous protein *S*-nitrosation. Therefore, whenever one uses the biotin switch assay to analyze the content of protein *S*-nitrosation, particularly when analyzing the level of endogenous *S*-nitrosation, ascorbate treatment should be used in parallel for both the sample of interest and the negative control. In this way, the effect of ascorbate on the degree of biotinylation is accounted for and qualitative conclusions can be drawn by comparison with the negative control.

Acknowledgments

The authors thank Dr. S. Perrett of this Institute for editing the manuscript. Thanks to Pei-wei Han for technical assistance and to Tiepeng Wang, Xixi Zhou, and Lin Li for their valuable advice. The work is supported by the National Natural Science Foundation of China (No. 30270352).

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