Published in final edited form as:

Anal Biochem. 2008 December 15; 383(2): 265–269. doi:10.1016/j.ab.2008.08.024.

A novel approach to enhance antibody sensitivity and specificity by peptide cross-linking

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Abstract

Most current techniques employed to improve antigen-antibody signals in western blotting and in immunohistochemistry rely on sample processing prior to staining (e.g. microwaving) or using a more robust reporter (e.g. a secondary antibody with biotin-streptavidin). We have developed and optimized a new approach intended to stabilize the complexes formed between antigens and their respective primary antibodies by cupric ions at high pH. This technique improves the affinity and lowers cross-reactivity with non-specific bands of ~20% of antibodies tested (5/25). Here we report that this method can enhance antigen-antibody specificity and can improve the utility of some poorly reactive primary antibodies.

Keywords

Western blot; antibody; sensitivity; specificity; Biuret reaction

Introduction

The antigen-antibody complex is a critical interaction exploited in a range of fundamental immune-based detection technologies such as immunoblotting, immunohistochemistry, immunoprecipitation and flow cytometric analyses. Yet antibody generation is something of a gamble, with no guarantee for the sensitivity or selectivity of an antibody raised against an epitope from a protein of interest, and whether the antibody will be effective across all desired techniques. Improving the performance of antibodies to enhance detection and signal-to-noise ratios continues to be a challenge in many research technologies. Several methods have been developed to improve antibody performance, most of which involve sample processing prior to application of the antibody (e.g. microwaving or albumin blocking) or using a more robust secondary antibody reporter that conjugates to the primary antibody (such as biotin-streptavidin and a range of chemiluminescent substrates that improve signals produced by horseradish peroxidase). However, little attention has been paid to enhancing the primary antibody application.

Cupric ions generate complexes between peptide backbones in an alkaline environment (Fig. 1A), in a reaction termed the biuret reaction which is the basic reaction underlying the measurement of protein concentrations [1]. We hypothesized that since this reaction cross-links two peptides, it could increase or stabilize the affinity between epitopes and antibodies,

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and thus could potentially be used to enhance antigen-antibody signals. After several trials and adjustments, we developed a novel approach to enhance antigen-antibody signals by modulating the affinity between epitopes and primary antibodies. This method has enabled us to enhance the signals of antibodies generated against several membrane-bound proteins, which had previously been poorly reactive or non-reactive. We believe that this basic technology will open new related approaches to enhance antigen-antibody reactions in a variety of fields and offers a promising alternative to improve the reactivity and usefulness of some antibodies that were previously poorly or nonreactive.

Materials and Methods

Cells and Antibodies

Murine nonagouti black (melan-a) melanocytes were derived from C57BL/6 mice and were a kind gift of Prof. Dorothy C. Bennett (London, UK) [2]. Murine melanocytes mutant at the SLC45A2 locus (hereafter termed uw-mutant) were derived in our laboratory from C57BL/6J mice with a genotype of uw/uw [3]. Melanocytes were cultured in RPMI 1640 Medium (Gibco BRL, Rockville, MD, USA) containing 5% fetal calf serum, 2 mM glutamine, 100 μ M 2-mercaptoethanol, 2.8 μ g/ml sodium bicarbonate, 48 nM 12-O-tetradecanoyl-phorbol-13-acetate (Sigma, St Louis, MO, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco BRL). Antibodies used, their epitopes, species, sources, etc, are listed in Table 1. Anti-rabbit IgG horseradish peroxidase-linked (whole antibody) was purchased from GE Healthcare (Buckinghamshire, UK). Normal goat serum was from Vector (Burlingame, CA). Alexa Fluor 594 F(ab')₂ fragment of goat anti-rabbit IgG (H+L) 2 mg/ml was from Invitrogen.

Reagents

Reagents for the biuret reaction included an alkaline buffer and a 0.16 M copper sulfate solution. The alkaline buffer contains 0.625 M NaOH, 0.70 mM sodium potassium tartrate and 1.1 M HCl (where indicated, the pH was adjusted with 1N HCl or 1N NaOH). These reagents were mixed in a ratio of 1 ml alkaline buffer and 25 μ l copper sulfate. Sodium potassium tartrate and copper sulfate were purchased from Sigma.

Western blotting

Cell extracts were prepared using M-PER mammalian protein reagent (Pierce, Rockford, IL, USA) containing complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were measured using the BCA protein assay (Pierce). Cell extracts were mixed with 2X Tris-glycine SDS sample buffer (Invitrogen) supplemented with 5% 2-mercaptoethanol and were boiled for 5 min. Samples (15 μ g protein/well) were then separated on 10% SDS-polyacrylamide gels (Invitrogen) and were transferred electrophoretically to PVDF membranes (Invitrogen) using an XCell II Blot Module (Invitrogen). The blots were blocked in 5% nonfat dry milk in PBS-Tween for 1 hr at room temperature and then were incubated with primary antibodies diluted in 5% nonfat dry milk in PBS-Tween overnight at 4°C. After three washes with PBS-Tween, the blots were incubated in horseradish peroxidase-linked anti-rabbit antibodies (1:10000) (GE Healthcare) in 5% nonfat dry milk in PBS-Tween for 1 hr at room temperature. After three further washes with PBS-Tween, the immunoreactivities of antibodies were detected using an ECL-plus Western blotting Detection System (GE Healthcare) according to the manufacturer's instructions.

When signals were enhanced with the biuret reaction, the membranes were immersed for 15 min into the reagent mixed with 1 ml alkaline buffer and 25 μ l copper sulfate solution after the initial primary antibody application. After treatment with the biuret reagent and three more washes with PBS-Tween, a second treatment with the primary antibody was applied.

Results

To determine the ability of the biuret reaction to enhance antigen-antibody reactions (Fig. 1A), we prepared the reagents and optimized them specifically to work in the western blotting format at different pHs. We used melanocytes as a model system since they express a number of specific proteins against which a large number of antibodies have been generated. Extracts of melan-a melanocytes and of uw-mutant melanocytes were probed for SLC45A2 protein expression using the α PEP30 antibody, which was generated against a specific peptide in loop 3 of the cytoplasmic domain of SLC45A2. The expression of *SLC45A2* was confirmed at the mRNA level using RT-PCR to show that it is expressed in melan-a melanocytes but not in uw-mutant melanocytes (data not shown) as expected. Although α PEP30 recognized the immunizing peptide at high titer (data not shown), only a minor band corresponding to the SLC45A2 protein (~58 kDa) was detected by α PEP30 using a standard western blotting protocol and background staining was high (Fig. 1B, left). However, when the biuret reagents were applied between the first and second primary antibody application steps, background staining was lowered considerably and a much stronger band corresponding to the predicted size of the SLC45A2 protein was observed in the SLC45A2-expressing melan-a melanocyte extract, but not in the extract of uw-mutant melanocytes (Fig. 1B, right).

Next, we examined the application of the biuret reagents between different steps in the western blotting procedure to determine whether they would be more effective if used elsewhere in the protocol. The specific band corresponding to SLC45A2 was not detected when the reagents were applied immediately after the blocking step (prior to the application of the primary antibody, Fig. 2A) or after the primary antibody application step (Fig. 2B). Therefore, two incubations with the primary antibody and treatment with the biuret reagents between those two steps are required for the enhancement.

The biuret reaction depends both on cupric ions and on an alkaline environment. To assess the optimal pH to produce an enhancement of antibody-antigen signals, we performed western blotting using the biuret reagents at different pHs. Western blotting with α PEP30 at pHs from 7 to 12 showed that the specific band corresponding to SLC45A2 was enhanced most dramatically at pHs 11 and 12, and that the nonspecific background bands gradually decreased with increasing pHs (Fig. 3A). Experiments using the same protocol with the HMB45 monoclonal antibody (which reacts with the melanocyte-specific protein Pmel17) showed that the specific bands of Pmel17 (which represent different processed forms of Pmel17 [4]) recognized by that antibody gradually decreased at higher pHs, and were almost reduced to background at a pH of 12 (Fig. 3B).

Finally, we screened a variety of other antibodies to determine whether their reactivities would be similarly enhanced by this novel protocol. Among the 25 antibodies tested, 18 were against epitopes of membrane-bound proteins, 3 were against cytoplasmic proteins and 4 were against nuclear proteins. Of those 25 antibodies, 5 (20%) were enhanced by this method, 2 against SLC45A2 (α PEP29 and α PEP30) and 3 against SLC24A5 (Rb3589, Rb3590 and α NCKX5) were enhanced by this method (Table 1).

Discussion

In this study, we developed a novel method to improve antigen-antibody interaction and specificity by increasing the affinity/cross-linking between epitopes and antibodies. This method was intended to take advantage of the biuret reaction that forms complexes between two peptide backbones (epitope and antibody), and thus links those two proteins together.

We optimized this method using α PEP30, an antibody that we recently generated against a specific peptide of the murine SLC45A2 protein. That antibody reacted at a high titer against the immunizing peptide, yet only weakly detected the intact protein using conventional western blot procedures. SLC45A2 has 12 transmembrane domains and is thought to regulate pH in melanosomes [5]; the antibody was generated in rabbits against a specific peptide in cytoplasmic loop 3 of that protein. We then screened 24 other antibodies to determine what type(s) of antibody would be enhanced with this method. So far this technology has enhanced only 5 of the 25 different antibodies tested, two against different epitopes of SLC45A2 and three against different epitopes of SLC24A5, another solute carrier protein. Our speculation about why only antibodies to SLC45A2 and SLC24A5 are enhanced is that both SLC45A2 and SLC24A5 are thought to be regulators of pH and thus might have great pH resistance. Treatment at high pH is essential to perform the biuret reaction effectively, however high pH can inactivate antigens and antibodies that are not pH-resistant because of changes in their tertiary structures that result from the alkaline environment. However, not all antibodies to those two solute carriers were enhanced, nor was an antibody to yet another solute carrier, SLC7A11.

We are now trying to further develop this protocol to treat antigens with reagents to improve their pH resistance, and are exploring the potential to use other metal ions that do not require high pH conditions to cross-link peptides. Such protocols would increase the number of antibodies that could be enhanced with this method, though many other proteins exist that are highly pH stable and could benefit from this enhancement method immediately. The improvement in antibody specificity is mediated by copper ions, which are well known for their ability to form stable coordination complexes with the amide of peptide bonds, and a number of amino acid side-chain residues [6]. When the biuret reagent is applied, antibody signals are improved, suggesting that copper-peptide interactions are able to improve reactivity and specificity, though whether this occurs by coordinating and blocking 'non-specific' sites, or improving epitope binding has not yet been determined. An alternative mechanism for this effect might involve the fact that increased pH combined with divalent metal ions such as copper can cleave immunoglobulins [7] and thus might free the antigen-binding fragments to react with the antigens.

We believe that this novel technology will facilitate studies on the localization and functions of membranous proteins such as SLC45A2 and SLC24A5, which have been slowed by the lack of effective antibodies. It might prove useful for investigators to try this new technique on antibodies that previously showed little or no reactivity, since dramatic improvement is seen for antibodies where the technique works. Following further development of methods to protect antigens from disruption by high pH and/or for signal enhancement without diminishing reactivity for other antibodies, it is our hope that a method for improving antibody-antigen specificity and signal can be employed universally.

Acknowledgements

This research was supported by the Intramural Research Program of the National Cancer Institute at the National Institutes of Health. We thank Drs. Michael M. Gottesman (Bethesda, MD, USA), Steve Wilson (Bedfordshire, UK) and Nancy Mangini (Gary, IN, USA) for the gifts of antibodies to ABCB5, NKX5 and SLC24A5, respectively.

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Figure 1. Enhancement of antibody signal using the biuret reaction

(a) Schematic of the biuret reaction to form peptide-peptide complexes. Peptides containing more than 3 amino acid residues form complexes with cupric ions in an alkaline environment in the presence of sodium potassium tartrate. (b) Left – standard Western blot protocol using α PEP30 with extracts of melan-a melanocytes and uw-mutant melanocytes as positive and negative controls, respectively. Middle - Primary antibodies were applied twice in Western blotting procedure and the biuret reaction reagent was applied between the two primary antibody applications as noted. Right - A band corresponding to the SLC45A2 protein (~58 kDa) was clearly enhanced with the biuret reaction reagent.

Figure 2. Application of biuret reaction after blocking and primary antibody application steps
(a) Left – standard Western blot as detailed for Figure 1. Middle – scheme showing the biuret reaction application after the blocking step. Right – Western blot after the biuret reaction application after the blocking step; no enhancement of the band corresponding to the SLC45A2 protein was noted.
(b) Left – standard Western blot as detailed for Figure 1. Middle – scheme showing the biuret reaction application after primary antibody application step but without a second primary antibody application; again, no enhancement of the band corresponding to the SLC45A2 protein was noted.

Figure 3.

Western blotting with enhancement using the biuret reaction at different pHs

(a) Western blotting with the α PEP30 antibody using the optimized biuret reaction shown in Figure 1. The intensity of the band corresponding SLC45A2 protein (~58 kDa) was increased at pH 11.0, and unspecific bands were decreased at higher pHs. (b) Western blotting with the HMB45 antibody; the intensities of bands recognized by HMB45 were decreased at pH 12.0.

Table 1

Summary of Antibody/Antigens Tested in this study.

Antibody	Antigen	Epitope	Species ¹	Localization	Pre ²	Post ²	Result	Ref	Source
aPEP17	P	peptide-cyto loop3	rabbit	membrane	±	±	no effect	[8]	Hearing laboratory
aPEP18	P	peptide-cyto loop5	rabbit	membrane	±	±	no effect	[8]	Hearing laboratory
aPEP35	P	peptide	rabbit	membrane	+++	++	no effect	[9]	Hearing laboratory
aPEP36	P	peptide	rabbit	membrane	±	±	no effect	[9]	Hearing laboratory
aPEP28	SL C45A2	peptide-C-terminus	rabbit	membrane	±	±	no effect	[9]	Hearing laboratory
aPEP29	SL C45A2	peptide-N-terminus	rabbit	membrane	±	+++	enhanced	[9]	Hearing laboratory
aPEP30	SL C45A2	peptide-cyto loop3	rabbit	membrane	±	+++	enhanced	[9]	Hearing laboratory
aPEP37	SL C45A2	peptide-N-terminus	rabbit	membrane	±	±	no effect	[9]	Hearing laboratory
aPEP38	SL C45A2	peptide-cyto loop5	rabbit	membrane	±	±	no effect	[9]	Hearing laboratory
OCTN1	SL C22A4	peptide-N-terminus	goat	membrane	±	±	no effect	[10]	Santa Cruz
207	SL C24A5	peptide-unspecified	rabbit	membrane	±	±	no effect	[11]	Mangini laboratory
Rb3589	SL C24A5	peptide-cyto loop	rabbit	membrane	±	++	enhanced	[12]	Wilson laboratory
Rb3590	SL C24A5	peptide-C-terminus	rabbit	membrane	±	++	enhanced	[12]	Wilson laboratory
aNCKX5	SL C24A5	peptide-C-terminus	rabbit	membrane	±	+++	enhanced	[12]	Wilson laboratory
aSLC7A11	SL C7A11	peptide-N-terminus	rabbit	membrane	+++	+	no effect	[13]	Novus Biologicals
aABCB5	ABCB5	peptide-cyto loop3	rabbit	membrane	±	±	no effect	none	Gottesman laboratory
aPEP7	TYR	peptide-C-terminus	rabbit	membrane	+++	+++	no effect	[14]	Hearing laboratory
aPEP8	Dct	peptide-C-terminus	rabbit	membrane	+++	+++	no effect	[15]	Hearing laboratory
Anti-SOX13	SOX13	peptide-N-terminus	rabbit	nucleus	±	±	no effect	none	Sigma
Anti-MDM4	MDM4	peptide-unspecified	rabbit	nucleus	++	±	no effect	none	GeneTex
Anti-CHD5	CHD5	recombinant protein	rabbit	nucleus	±	±	no effect	none	Strategic Diagnostics
Anti-ELF3	ELF3	peptide-unspecified	rabbit	nucleus	++	±	no effect	[16]	Aviva System Biology
HMB-45	Pmel17	intact protein	mouse	cytoplasm	+++	+	no effect	[17]	Dako Corp
aPEP24h	Pmel17	peptide-internal	rabbit	cytoplasm	±	±	no effect	none	Hearing laboratory
aPEP26h	Pmel17	peptide-internal	rabbit	cytoplasm	±	±	no effect	none	Hearing laboratory

¹ All antibodies are polyclonal, except for HMB-45 which is monoclonal.

² **Pre** – indicates reactivity prior to the antibody amplification; **Post** - indicates reactivity after antibody amplification. Scale ranges from – (no reactivity) to ++++ (strong reactivity)