Published in final edited form as:

Biochim Biophys Acta. 2015 June ; 1854(6): 592–600. doi:10.1016/j.bbapap.2014.10.011.

Immuno-proteomics: Development of a novel reagent for separating antibodies from their target proteins

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Abstract

Immunoprecipitation (IP) is a widely used technique for identifying the binding partners of the target proteins of specific antibodies. Putative binding targets and their partners are usually in much lower amounts than the antibodies used to capture these target proteins. Thus antigen identification using proteomics following IP, is often confounded by the presence of an overwhelming amount of interfering antibody protein. Even covalently linking antibodies to beads is susceptible to antibody leaching during IP. To circumvent this interference, we describe here a reagent, called Biotin-CDM that reversibly tags all potential target proteins in a cell lysate with biotin. The presence of biotin coupled to the target proteins allows for a secondary separation step in which antibodies are washed away from the reversibly biotinylated target proteins by binding them to an Avidin-coupled matrix. The captured target proteins are released from the Avidin matrix by reversing the Biotin-CDM link, thus releasing a pool of target proteins ready for further proteomic analysis compatible with 2D-Electrophoresis. Here, we describe the synthesis and characterization of Biotin-CDM. We also demonstrate Biotin-CDM's use for immunoprecipitation of a known antigen, as well as its use for capturing an array of proteins targeted by the autoantibodies found in the serum a patient suffering from rheumatoid arthritis. The use of this reagent allows one to combine immunoprecipitation and 2D-Difference gel electrophoresis, overcoming the current limitations of Serological Proteome Analysis (SERPA) in discovering autoantigens.

Keywords

immuno-proteomics; serological proteomics; difference gel electrophoresis (DIGE); reversible biotin; immunoprecipitation

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1. Introduction

Immunoprecipitation (IP) is a ubiquitously used method in biomedical research where antibodies are first bound to a solid matrix, such as Protein-A or Protein-G beads, and then used to capture the antibodies' target proteins from cellular lysates or bodily fluids. An extension of IP is co-immunoprecipitation (co-IP), which is commonly used to capture the binding partners of target proteins via their binding to previously characterized antibodies. Co-IP is primarily used to explore biological processes such as cell signaling and regulation by studying protein-protein interactions. Probing human cell proteome using patient sera is referred to as Serological Proteome Analysis (SERPA), is very useful for identifying therapeutic and diagnostic targets (Klade et al., 2001). This approach uses 2D-western blots to identify autoantigens in the case of cancer and autoimmune diseases (Gunawardana and Diamandis, 2007). Sample containing autoantigen is run in three identical 2 dimensional electrophoresis(2DE) gels. Two gels are probed in western blot using healthy and patient anti-sera respectively. Using the western blot results as a map, the corresponding protein spots are cut from the third gel. First of all, there is a considerable amount of gel-to-gel variability between 2DE experiments. Secondly, low-abundant autoantigens suffer from poor identification using a cut-map from a different 2DE gel. Although multiple strategies have been developed (Cottrell et al., 2012) to address these issues in identifying autoantigens using SERPA, lack of reproducibility and inability to identify low abundant proteins persist as severe limitations to this approach. Immunoprecipitation enriches low abundant proteins and would be the ideal tool for identifying antigens. Unfortunately, proteomic analysis of immunoprecipitated samples also has very significant limitations.

Proteomic analysis of IP and co-IP target proteins is often complicated by the presence of variable amounts of immunoglobulins and other background proteins derived from the anti-sera or the target-containing cellular extracts. The background proteins are proteins that bind non-specifically to the antibody or anti-sera beads (Howell et al., 2006). Addition of competing proteins such as BSA helps reduce non-specific proteins from binding the antibody beads. Increasing the salt concentration, number of washes and adding detergent to the wash steps help in reducing the non-specific proteins from binding (Harlow and Lane, 1999). These are simple solutions to the non-specific protein problem.

However, the larger problem is the release of abundant immunoglobulins from the affinity beads, which can eclipse the detection of low-abundance target proteins. Generally, high concentrations of denaturants, such as urea or SDS, are used to elute proteins bound to antibodies (Harlow and Lane, 1999). Under these strong denaturing conditions, antibodies leach from the resin, contaminating the eluted sample (Yang et al., 2008). This poses a serious problem for mass spectrometry (MS)-identification of low abundance target proteins. Currently, there is no simple, generic solution to eliminate antibodies from the eluate. Typical solutions to this problem are to either covalently cross-link the antibodies directly to the polymer bead matrix or to covalently cross-link the antibodies to Protein-A/G beads, via protein-protein crosslinking. Directly conjugating antibodies to a resin results in the random orientation of the antibodies on the matrix, potentially reducing the efficiency of the antibody-antigen interaction. Also, only purified antibodies can be directly linked to the resin, limiting ones options when working with patient anti-sera. Cross-linking of the

antibodies to Protein-A/G beads can be a tedious process that requires optimization for each serum or antibody used. Over cross-linking risks losing antibody reactivity, while under cross-linking again can lead to variable amounts of antibody leaching from the beads during target elution. Antibodies and anti-sera are usually precious reagents. One is often loath to expend this limited material optimizing the cross-linking protocol. Since antigen affinity is very specific to each antibody, the cross-linking of each anti-sera to be tested needs to be optimized. This is not practical when dealing with patient sera for SERPA.

Seeking a way to overcome this critical limitation of IP, we describe here a method that allows one to efficiently separate antibodies from their target proteins. Since antibodies are derived from a huge array of anti-sera, which are usually limited in supply, we decided to focus on the source of target proteins. Target proteins are typically derived from commonly used, tissue culture cell lines, which can be grown in abundance, or from tissue samples. While tissue samples may not be as abundant as cultured cell lines, they are certainly more easily obtained than specific anti-sera. To discriminate between the target proteins and the antibodies that bind them, one needs to attach a suitable affinity reagent to the target proteins. Ideally, the affinity tag should be able to function properly under the harsh conditions used to elute proteins from antibody beads. Biotin is arguably the best candidate for such an affinity tag since it binds to Avidin and Avidin derivatives, such as NeutrAvidin, with exceptionally high affinity under quite harsh conditions. The ultimate goal of this method is to subject the isolated proteins for proteomic analysis, which may be affected by the presence of the affinity tag. This is particularly true for two-dimensional electrophoresis (2DE) (O'Farrell, 1975), gel-based proteome analysis methods, such as DIGE (Unlu, 1999). The tag is expected to alter the mass and charge of the targets. To avoid this effect, we designed a reversible, biotin-labeling reagent, called Biotin-CDM. We show here: that Biotin-CDM can be used to extensively tag cellular protein lysates, that these tagged, target-proteins can be captured by their cognate antibodies, that the target proteins can be separated from their cognate antibodies and that the tag can be efficiently removed from the target proteins for 2DE, gel-based proteome analysis.

2. Materials and methods

2.1. Materials

The hydroxysuccinimidyl ester of 2-propionic-3-methylmaleic anhydride (CDM-NHS ester) was custom synthesized by GLSynthesis Inc (Worcester, MA). Biotin was purchased from AK Scientific corporation (Union City, CA). 2,2'-(Ethylenedioxy) diethylamine was purchased from Tokyo Chemical Industry Company of America (TCI America, Portland, OR). The target compound was purified on a Buchi Corp. Sepacore (New Castle, DE), MPLC system and further analyzed by UPLC (Waters Corp., Acquity, Milford, MA). ¹H-NMR and COSY experiments were performed on a Bruker Corp. 300 MHz instrument (Billerica, MA). ESI-MS analysis was performed on a Finnigan LCQ (Thermo Fisher Scientific, Waltham, MA). Bovine Serum Albumin, Fraction V (BSA) was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA) and Alcohol dehydrogenase (ADH) was purchased from Sigma Aldrich Corp., St. Louis, MO). Minimal DIGE dyes Cy3 and Cy5 were obtained from GE Healthcare (Pittsburgh, PA). Amicon Ultra 4 ml 10K

NMWL spin filters were purchased from EMD Millipore (Billerica, MA). High capacity NeutrAvidin was purchased from Pierce (Thermo Fisher Scientific, Waltham, MA). Protein-A Sepharose CL-4B was purchased from GE Healthcare (Pittsburgh, PA). 18 cm pH 3–10NL IPG strips were purchased from BIO-RAD Corp. (Hercules, CA).

N-Boc-2,2'-(ethylenedioxy)diethylamine, *BocNH-2p-NH₂* was prepared according to the procedure of Lee et. al. (Lee et al., 2007). The starting material for this reagent, 1,2-Bis(2-aminoethoxy)ethane was purchased from TCI America Inc. Biotin-NHS ester was purchased from Chem-Impex International, Inc. Diethylisopropylamine was purchased from Aldrich Chemical Company, Inc. Ethyl acetate, methanol, acetonitrile and Dimethylformamide (DMF) were purchased from EMD Millipore. Silicagel 60 Å, Premium R_f was purchased from Sorbend Technologies. All the other reagents were purchased from Fisher Scientific. MPLC chromatography was performed on a Buchi Sepacore system using RP-18 (SMT Bulk-C18) manufactured by Separation Methods Technologies.

To prepare N-Boc-2,2'-(ethylenedioxy)diethylamine, a solution of 150 mL of MeOH with cooling at 0 °C, HCl gas (17 g) was added with stirring for 15 min. The mixture was stirred for 15 min at room temperature and was carefully added to N,N'-bis(aminoethoxyethane) (67g, 0.466 mol) at 0 °C. The mixture was stirred for 15 min at room temperature before adding 50 ml of H₂O and stirring for another 0.5 h. To the solution (BOC)₂O (101 g, 0.466 mol) in 200 ml of MeOH was added at room temperature for 10 min, and the resultant solution was stirred for 1 h. The mixture was concentrated in vacuo. Unreacted diamine was removed by diethyl ether (300 mL × 2). The residue was treated with 2 N NaOH (500 ml) solution. The product in the organic layer was extracted with CH₂Cl₂ (300 ml × 3). The combined extracts were washed with 300 ml of brine, dried over anhydrous MgSO₄, and concentrated in vacuo to yield 64.6 g (87%) of mono-BOC product as a colorless oil with more than 97% purity by HPLC.

2.2. Chemical synthesis

Synthesis of Biotin-2p-NH₂ hydrochloride 1 (Fig. 2)—N-Boc-2,2'-(ethylenedioxy)diethylamine *BocNH-2p-NH₂* (992 mg, 4 mmol) was added to biotin-NHS ester (1.36 g, 4 mmol) dissolved in 5 ml of dry DMF. Diethylisopropylamine (0.7 ml, 4 mmol) was added and the reaction mixture was stirred at 50 °C for 2 hrs. The reaction mixture was concentrated under vacuum. The residue was dissolved in ethyl acetate (70 ml) and washed with 1 M citric acid, water and 1 M bicarbonate. The organic phase was dried over sodium sulfate and concentrated to give 1.28 g (67 % yield) of an oily residue.

The TLC (silica gel) showed one spot at R_f=0.2 (eluent: ethyl acetate/20% methanol) that stained positive for biotin.

Biotin-2p-NH-Boc (1 g, 2.1 mmol) was dissolved in methanol (5 ml) and 1 N HCl (5 ml) was added. The reaction was stirred overnight at room temperature. TLC showed the removal of the Boc group. The reaction mixture was concentrated and the resulting solid used as such in the next reaction step. ESI/positive M⁺ 375.3 g/mol

Synthesis of Biotin-CDM 4—Biotin-2p-NH₂ hydrochloride **1** (2.2g, 5.3 mmol) was dissolved in 1M Triethylammonium bicarbonate buffer pH 8.5 (10 ml). CDM-NHS ester **2** (0.75 g, 2.65 mmol) dissolved in dry dioxane (5 ml) was added dropwise under stirring at room temperature. Stirring was continued for 1 hr. The reaction mixture was concentrated to a volume of 1 ml. 2N HCl (5 ml) was added to hydrolyze **3** and the reaction mixture was stirred for 30 minutes. The reaction mixture was separated by MPLC chromatography on a Sepacore system on RP-18 with acetonitrile/water/0.1% TFA as mobile phase using a step-gradient. The product eluted at 20% acetonitrile (monitoring at 254 nm). The product fractions were analyzed by UPLC (Waters), RP-18 column, acetonitrile/water/0.1% TFA, 0–0.5min, 0 % acetonitrile, 0.5 min–3 min 0–100% acetonitrile linear gradient, run time 5 min, monitoring at 240 nm and 256 nm. Pure fractions at R_f 1.72 were collected and concentrated to yield 486 mg (34%) of the colorless resinous product **4**.

¹H-NMR (MeOD, 300 MHz) 4.51 (1H,dd, J=7.8 Hz, 4.4 Hz), 4.32 (1H, dd, J=7.8 Hz, 4.4 Hz), 3.63 (4H,m, *2p-linker*), 3.34 (4H,m, *2p-linker*), 3.22 (1H,m), 2.94 (1H, dd, J= 7.8 Hz, 12.6 Hz), 2.78 (2H,t, J=7.2Hz, *CDM*), 2.75 (1H,d, J=12.6 Hz), 2.54 (2H,t, J=7.2 Hz), *CDM*), 2.25 (2H, t, 12.5 Hz, 2.08 (3H,s, *CDM*), 1.68 (4H,m), 1.46 (2H,m).

2.3. Binding and release of Biotinylated proteins

Commercially available pure proteins BSA and ADH were used to test the binding and release of biotinylated proteins from NeutrAvidin resin. 1 mg of BSA in 200 µl of Wash Buffer (WB - 20 mM HEPES, 2 M urea, 2% CHAPS, 10 mM DTT, pH 8.0) plus 150 mM NaCl was labeled with 10 nmol of minimal Cy3-DIGE dye (in 5 µl DMF) for 15 minutes on ice. This reaction was then made up to 0.5 ml in WB and Biotin-CDM at 55 mM in DMF was added at a 1- to 5-fold mass ratio over protein and further reacted for 30 minutes on ice. Quencher (2 µl of 5 M methylamine, 10 mM HEPES-HCl pH 8 (Minden, 2012; Viswanathan et al., 2006) was added to halt the labeling reaction. Similar labeling reaction was carried out to yield Cy5-, Biotin-CDM-ADH. Excess dye and Biotin-CDM were removed using Amicon 10K NMWL spin filters with five 10-fold washes of WB plus 150 mM NaCl. Cy3/5-, Biotin-CDM-labeled proteins (5 µg BSA or 100 µg ADH) were bound to 200 µl of 50% slurry of high capacity NeutrAvidin slurry in WB plus 150 mM NaCl for 30 minutes at 4 °C by rotating end-over-end. The flow through was collected by centrifugation at 100 X g for 30 s. The NeutrAvidin beads were washed with a sequence of 0.5mL solutions containing WB plus 500 mM, 150 mM, 50 mM and 5 mM NaCl. This sequence was used to first reduce non-specific binding and to prepare the beads for a low-salt elution step, which is required for subsequent proteome analysis. The bound proteins were released from the linked Biotin-CDM and eluted from the NeutrAvidin beads using 300 µl of low pH elution buffer (EB 20 mM citrate, 8 M urea, 2% CHAPS, 10 mM DTT, 2 mM NaCl pH 3.7) by rotating end-over-end at 4 °C for 30 minutes. This elution step was repeated once more to recover maximum protein. To assess the efficacy of binding and release, a 2% volume of each fraction was loaded on to a 12% SDS-PAGE gel. Fluorescence quantification was done with ImageJ. Care was taken to subtract the background fluorescence signal in order to determine the fraction of input protein bound and released from the beads.

2.4. Co-immunoprecipitation

HeLa protein lysate preparation—T2-HeLa cells lysates were prepared by first rinsing the 80% confluent 10 cm culture plates with 10 ml cold DPBS twice. The cells were lifted in 10 ml buffer containing 250 mM Sucrose, 20 mM HEPES and 1 mM EDTA pH 8.0. The cells were pelleted at 930 X g for 5 minutes. The cell pellet was washed in the same sucrose buffer and pelleted at 930 X g for 5 minutes. The pellet volume was estimated and resuspended in 3 volumes of lysis buffer (LB) containing 20 mM HEPES pH 8.0, 0.3 M NaCl, 1 mM EDTA, 10 mM DTT, 5 mM CaCl₂, protease inhibitor (10 µl/ml) and disrupted using a 3 ml ball homogenizer. Protein concentration was measured using Bradford reagent (Sigma). Protein extract (1 mg in 1ml LB) was labeled with 10 nmol minimal Cy3-DIGE dye (resuspended in 4 µl DMF) for 15 minutes on ice. For biotinylated samples, 2 mg Biotin-CDM (from 55mM stock in DMF) was added to 1mg of protein extract for 30 minutes on ice and then 10 µl of Quencher was added to stop the labeling reaction.

Antisera/Antibody beads preparation—Rabbit anti-P115 polyclonal antibody (10 µl, a gift from Dr. Adam Linstedt) or RA anti-sera from an anonymous donor (30 µl) were bound to Protein-A Sepharose CL-4B (2.5 mg of dry beads) in 0.5 ml PBS overnight at 4 °C. The antibody/anti-sera beads were then washed with 0.5 ml PBS thrice. The prepared antibody/anti-sera beads were resuspended in 0.5 ml PBS. Cy5-DIGE dye (0.83 nmol resuspended in 1 µl of DMF) was added to the slurry and the labeling reaction was incubated at 4 °C for 15 minutes by rotating end-over-end. 1 µl Quencher was added to stop the labeling reactions.

Immunoprecipitation—IP was carried out by end-over-end mixing the Cy-3 labeled protein extract with the Cy5-labeled antibody/anti-sera beads for 2 hours at 4 °C. The beads were washed thrice with 500 µl of a solution containing: 10 mM HEPES pH 8.0 and 100 mM NaCl. To elute the proteins bound to the Protein-A beads, 100 µl of a solution containing: 10 mM HEPES pH 8.0, 7 M urea, 2% CHAPS, and 10 mM DTT, was added to the beads and mixed end-over-end for 30 minutes at 4 °C. The beads were spun at 100 X g for 30 s to elute the bound proteins.

Separation of biotinylated target proteins from antibodies—The eluate from the Protein-A beads was diluted with three volumes of a solution containing 50 mM HEPES pH 8 and 150 mM NaCl, lowering the urea concentration to 2 M in the binding reaction. The diluted eluate was incubated with 200 µl of 50% slurry of high capacity NeutrAvidin in WB plus 150 mM NaCl. The same wash and elution conditions were used as described in section 2.3. The different fractions of the procedure were analyzed on a 12% SDS-PAGE gel (the loads were 2% for all fractions, except the final NeutrAvidin eluate, which was 20%).

2.6. DIGE and Two-dimensional electrophoresis

2DE was performed using 18 cm pH 3–10NL IPG strips (Bio-Rad) as previously described (Viswanathan et al., 2006). The strips were rehydrated with a rehydration buffer containing 0.2% pH 3–10NL Bio-lyte (Bio-Rad) for 18 hours and IEF was carried out on a Protean IEF instrument (Bio-Rad). The second dimension, SDS-PAGE gel was composed of a 12% resolving gel run on a Protein II xi electrophoresis apparatus (Bio-Rad). Transfer from IEF to the second dimension was carried out using in-gel equilibration protocol (Van et al.,

2014). The gel was imaged and quantified using an in-house-built fluorescent gel imager (Sellers et al., 2007). Gel images were analyzed in ImageJ. Dust specks and “hot” pixels were removed from the images by using the “Process>Noise>Remove Outlier” function of ImageJ. Image contrast for “normal” contrast images was set with a single round of the “Image>Adjust>Brightness/Contrast>Auto” function, which sets the minimum and maximum intensity to the 5th and 95th percentile, respectively. “High” contrast images were set manually to visually enhance the contrast as to view low intensity signals. The intensity ranges of these “normal” and “high” contrast images will be discussed in the results section. Protein spot detection and analysis was performed using DeCyder software (GE Healthcare).

3. Results and discussion

3.1 Strategy

The goal of effort was to create a coupling reagent with two features: one end of the reagent should be an affinity tag that could withstand relatively harsh wash conditions, while the other end should form a reversible, covalent bond with target protein lysates, thus coupling the affinity tag to the protein pool. Biotin was chosen because of its very strong affinity to Avidin and its ability to withstand denaturants, such as urea and SDS and reducing reagents, such as DTT and 2-mercaptoethanol.

There were two classes of coupling moieties to consider: one reacts with cysteine sulfhydryls and the other reacts with primary amine groups of the lysines and amino termini. Since the potential target proteins are both cytoplasmic and extra cellular, it is important to not alter the disulfide state of the lysates. Therefore, we chose a primary amine-reactive moiety over sulfhydryl reactive compounds. Maleic anhydride derivative represent a class of pH reversible, amine-reactive compounds (Atassi and Habeeb, 1972; Perham, 1968). Maleic anhydride forms covalent bonds with amine under mildly basic conditions that can be reversed under mildly acidic conditions (Nieto and Palacian, 1983), however the reversal requires hours of exposure to complete the reaction. The addition of alkyl groups to the 2 and 3 positions of the maleic anhydride ring greatly accelerates the amide bond reversal. Thus, the overall design of the reagent was to link Biotin to 2,3 alkyl maleic anhydride, which we refer to as Biotin-CDM (Fig. 1A). Combining CDM with protein mixtures under mildly basic conditions couples the Biotin-CDM to the protein's lysine residues. Lowering the pH to below 5 reverses this linkage, releasing the protein in an unmodified state, ready for further proteomic analysis.

The general scheme for using Biotin-CDM to separate target proteins from their capturing antibodies is cartooned in Figure 1B. Protein-A beads first bind either purified antibodies or antibodies found in anti-sera. The immobilized antibodies bind to their cognate Biotin-CDM-tagged target proteins. After washing to remove unbound proteins, the beads are eluted with a denaturant solution, which strips the captured Biotin-CDM-tagged target proteins, as well as the immobilized antibodies. Since the elution is performed under denaturing conditions, the antibodies no longer bind the target proteins. This eluate is then combined with NeutrAvidin beads, capturing Biotin-CDM-tagged proteins, but not the untagged antibodies. After washing, the bond between Biotin-CDM and the target protein is

reversed by incubation in a mildly acidic solution, thus releasing the target proteins, the Biotin-CDM remains bound to the NeutrAvidin beads.

3.2 Synthesis of Biotin-CDM

The synthesis of Biotin-CDM was carried out in two steps (Fig. 2). First, two equivalent of Biotin-2p-NH₂, which was derived from coupling of N-Boc-2,2'-(ethylenedioxy) diethylamine (Lee and Ha, 2007) and biotin-OSu (von Grünigen et al., 1991) followed by Boc-deprotection, were reacted with one equivalent of the hydroxysuccinimidyl ester of 2-propionic-3-methylmaleic anhydride (CDM-NHS ester) in triethylammonium carbonate buffer, pH 8.5. Two equivalents of Biotin-2p-NH₂ was used in this first step because both the NHS and maleic anhydride moieties of CDM-NHS were capable of reacting with Biotin-2p-NH₂. Since the CDM amide is reversible, while the amide formed through the NHS ester is stable, the resulting bis-biotinylated intermediate was treated with 1N HCl to release the reversible CDM-bound Biotin-2p-NH₂ moiety. The target compound was purified by RP-MPLC on a preparative C-18 column using a step acetonitrile gradient in an aqueous solution (0.1% TFA) and characterized by ¹H-NMR.

3.3 Optimizing the capture and release of Biotin-CDM-labeled proteins

With the Biotin-CDM in hand, we wished to optimize the capture and release of Biotin-CDM-tagged proteins on NeutrAvidin beads. NeutrAvidin was chosen because it has the highest biotin binding capacity of commercially available Avidin-derivative matrices. There were four optimization criteria: (1) determine the ratio of Biotin-CDM to protein for an excess of NeutrAvidin beads to capture the majority of protein in a solution; (2) determine the binding capacity of NeutrAvidin for Biotin-CDM-tagged protein; (3) determine the optimal conditions for maximum reversal of the CDM linkage and release of protein from NeutrAvidin beads; and (4) assess the fraction of protein species captured and released from NeutrAvidin beads relative to the starting protein lysate.

Purified BSA and ADH were first fluorescently tagged with either Cy3-NHS or Cy5-NHS minimal DIGE dyes to aid in quantification. These proteins were then tagged with Biotin-CDM. It was essential to remove as much free Biotin-CDM as possible so that the free Biotin-CDM would not compete with Biotin-CDM-tagged protein for NeutrAvidin binding. Therefore, unbound fluorescent dye and biotin-CDM were removed by spin-dialysis over five rounds of dilution and concentration with binding buffer. The resulting proteins were used for method optimization as follows.

To optimize the extent of Biotin-CDM labeling for binding to NeutrAvidin beads, our goal was not to obtain 100% binding since over-labeling the proteins may have adverse effects on protein solubility and accessibility to antibody binding. We therefore aimed for the amount of Biotin-CDM labeling that allowed for 60–80% binding. The weight-to-weight ratio of Biotin-CDM to 5 µg of Cy3- labeled BSA was varied from 0.5 to 2 (Fig. 3A). All Biotin-CDM labeling ratios of 0.5 to 2 produced capture efficiencies within the target range. The ratios between 1:1 and 2:1 gave the best capture extents for BSA. Increasing the Biotin-CDM to protein ratio beyond 2 lead to a decrease in protein solubility. Therefore, the 2:1 ratio was chosen as the optimal coupling ratio.

To determine the binding capacity of NeutrAvidin for Biotin-CDM-tagged protein, we titrated 1:1 Biotin-CDM-tagged BSA relative to 100 μ l of packed NeutrAvidin beads. The upper limit of protein binding for this given amount of NeutrAvidin, was found to be 100 μ g (Fig. 3B). This amount of protein is also typical of the amount used for 2D DIGE experiments.

A variety of conditions were tested for optimizing the scission of the Biotin-CDM-protein bond and the release of protein from the NeutrAvidin beads. The most important considerations were to: determine the optimal pH required to reverse the CDM linkage without adversely affecting protein solubility and finding the balance of salts, buffers, denaturants and detergents that are compatible with follow-on proteomics analysis. For samples that will be analyzed by 2D DIGE, the optimal elution buffer was found to be 20 mM citrate, 8 M urea, 2% CHAPS, 10 mM DTT, 2 mM NaCl pH 3.7. Incubation of the Biotin-CDM tagged ADH bound to NeutrAvidin beads for 30 minutes at 4 °C provided nearly 100% recovery of the bound protein (Fig. 3C). Figure 3C also demonstrates that this eluted material can be analyzed by conventional SDS-PAGE by mixing the eluate 1:1 with 2 X Laemmli sample buffer.

Finally, we used 2D-DIGE to assess the spectrum of biotin-CDM-tagged HeLa lysate proteins captured and released by NeutrAvidin beads relative to the input protein lysate. Cy-3-labeled, Biotin-CDM-tagged HeLa cell lysate (~50 μ g) that had been captured and released from NeutrAvidin beads was compared to an equal amount of Cy5-labeled HeLa cell lysate. The 2D-DIGE analysis demonstrated that the majority of protein species were retained throughout the Biotin-CDM tagging, NeutrAvidin binding and acidic reversal of the Biotin-CDM bond (Figs. 3D). Figure 3D shows a 2D-DIGE gel of 2:1 Biotin-CDM labeling of Cy5-labeled HeLa lysate versus Cy3-labeled HeLa control lysate that was not treated with Biotin-CDM. The overlay of the Cy3 (green) and Cy5 (red) channels shows that a very good representation of the majority of HeLa protein spots in the biotin-CDM tagged proteins that were captured by NeutrAvidin beads and released by reversing the Biotin-CDM linkage versus untreated Cy3-labeled proteins. Automated image analysis (DeCyder) of this 2D-DIGE gel showed that the 328 detected protein spots that ranged over two-orders of magnitude in abundance were represented in both images with a correlation value (R^2) of 0.91. The maximum fold-difference between treated and untreated for individual proteins within these samples was about ± 2.5 ; none of the protein spots detected in the untreated, control sample were absent from the Biotin-CDM captured sample (Fig. 3E). It is important to note that to perform this analysis the samples were extensively spin dialyzed with 10 kD NMWL devices to remove unbound Biotin-CDM, the consequence of which was a significant loss of protein, resulting in relatively under-loaded 2D gels, particularly in the lower molecular weight range < 20–30 kD. In spite of sub-optimal protein loads, the 2D-DIGE gels showed an excellent recovery of Biotin-CDM labeled proteins from the NeutrAvidin beads. Since even a single Biotin-CDM bound to a protein will alter the protein's migration on a 2DE gel, these results demonstrate that the Biotin-CDM is completely leased from the target proteins as evidenced by the perfect overlap between control and Biotin-CDM captured proteins.

All of the experiments described in this report were repeated at least three times, all with consistent results. These optimization data show that tagging protein lysates with Biotin-CDM yielded a representative pool of target proteins that can be efficiently captured and released under conditions that will allow for further proteome analysis. These conditions also allow for the removal of unwanted reagents such as salts, denaturants, detergents, and buffers which is important for the efficiency of isoelectric focusing, the first dimension of 2DE.

3.4 IP/co-IP and Biotin-CDM cleanup of a known protein antigen

To test the efficacy of Biotin-CDM as a method for removing antibody contaminants from an immunoprecipitation of a known antigen using a well-characterized antibody, we used anti-p115 antibody. p115 is a 115 kD protein with a single transmembrane domain near its C-terminus that resides in the Golgi membrane and is required for proper sorting of Golgi vesicles (Guo et al., 2008). HeLa cell lysates, which are known to express p115, were prepared in the standard lysis buffer augmented with 0.5% Triton X-100 to solubilize membrane proteins. The HeLa cell lysate (1 mg) was fluorescently labeled with Cy3-NHS and tagged with Biotin-CDM (2:1 labeling ratio of Biotin-CDM:protein). Rabbit anti-p115 anti-serum was mixed with Protein-A beads to immobilize the anti-p115 antibodies. After mild washing, the bound proteins were labeled with Cy5-NHS to monitor antibody abundance throughout the IP procedure. As a control, HeLa cell lysate was fluorescently labeled with Cy3-NHS, but not coupled to Biotin-CDM (Figs. 4A and A').

The control experiment, which lacked Biotin-CDM tagging, showed that most of the cell lysate passed directly through the anti-p115:Protein-A beads (Fig. 4A, α -bead flow thru). Elution of the anti-p115:Protein-A beads with a solution containing 8M urea released a large amount of Cy5-labeled antibody that hinders mass-spec identification of the target proteins (Fig. 4A, α -p115 eluate). Fig. 4A shows the gel image set at "normal" contrast (intensity range – 425 to 19631 counts), which prevents one from visualizing low abundance proteins. To visualize the presence HeLa proteins in the antibody bead eluate, "high" contrast image (intensity range – 425 to 966 counts) of the same gel is shown in Fig 4A'. At this enhanced intensity range one can detect a small amount of released Cy3-HeLa protein (green) and an overwhelming amount of Cy5-anti-p115 antibody (red) (Fig 4A', compare red and green signals in the α -p115 eluate lane). This material was combined with NeutrAvidin beads. When the proteins lacked Biotin-CDM, all of the Cy5-antibody and Cy3-HeLa protein was found in the bead flow through and wash. As expected, virtually no protein was detected in the eluate of this control experiment, indicating that without Biotin-CDM no protein binds to the NeutrAvidin beads- (Fig. 4A', Avidin eluate).

As expected, coupling of Biotin-CDM to the HeLa lysates gave a different result (Figs. 4B and B'). "Normal" contrast image (intensity range – 220 to 20091 counts) of the gel showed only the contents of the antibody beads and the cell lysate load and flow through (Fig. 4B, Cell lysate load and α -bead flow thru lanes). The antibody that leaked from the Protein-A beads in the eluate was not visible in this "normal" contrast image due to slight variation between experiments, but is seen in the "high" contrast image (intensity range - 220 to 1060 counts) of the same gel (Fig 4B'', α -p115 eluate). Increasing the image contrast allowed one

to visualize that many Cy3-HeLa (green) proteins were released from both the anti-p115:Protein-A beads and the NeutrAvidin beads (Fig. 4B', α -p115 eluate and Avidin eluate lanes). On the other hand, Cy5-antibody (red) was only detected in the Protein-A bead eluate, but only slightly detectable in the NeutrAvidin eluate (Fig. 4B', α -p115 eluate and Avidin eluate lanes). Thus Biotin-CDM effectively removed the vast majority of antibody from the anti-p115 eluate.

This experiment using a known antigen, was designed to perform both IP and co-IP. The anti-p115:Protein-A beads were only washed in low salt buffer to detect proteins bound to the target protein and its binding partners. Thus both specific and non-specific proteins may be bound to the antibody beads. Further IP experiments with more stringent washes will be required to assess the specificity of the bound proteins. One protein that was expected to bind throughout the experiment was p115. As opposed to boiling all of the antibody beads to load onto the SDS PAGE gel, only 20% fraction of the cleaned-up IP eluate was analyzed in the gel shown. This fraction of eluate was below the detection limit of the antibody using an immunoblot. However, magnifying the section of the 100 to 130 kD range of the gel and enhancing the contrast revealed 2 protein bands at ~115 kD (Fig. 4B''). A western blot performed on HeLa protein lysate using rabbit anti-P115 primary antibody (1:3000 dilution) and anti rabbit HRP conjugate (1:500 dilution) showed 2 protein bands (Fig. 4C) in the same position as seen in Fig 4B''. Further mass spectrometry analysis is required to confirm that this protein is indeed p115. These data demonstrate that this approach is effective at removing antibodies from IP and co-IP experiments and that the target proteins are effectively retained by the NeutrAvidin beads and released under mildly acidic conditions.

3.5 IP and Biotin-CDM cleanup of unknown serum antigens

To evaluate the suitability for the use of Biotin-CDM to cleanup target protein samples derived from patient anti-sera, we tested anti-serum from a patient suffering from rheumatoid arthritis. Understanding the range and identity of autoantigens targeted by a patient's immune system will be helpful in furthering our understanding of this common autoimmune disease. Antibodies from the serum of an anonymous rheumatoid arthritis (RA) patient were captured by Protein-A beads and labeled with Cy5-NHS so that the antibody proteins could be tracked through this procedure. The RA:Protein-A beads were challenged with Cy3-labeled, Biotin-CDM-tagged HeLa cell lysate. As was the case with anti-p115 antibodies, both Cy5-RA-antibodies and Cy3-cell lysate proteins were eluted from the Protein-A beads (Fig. 5A). This eluate was applied to NeutrAvidin beads. Also as shown previously, an abundance of Cy5-antibody flowed through the Protein-A beads, which was easily detected in the "normal" contrast SDS-PAGE gel image (intensity range – 848 to 21402) (Fig. 5A). "High" contrast image (intensity range – 163 to 2666) was required to observe the abundance of Cy3-labeled HeLa lysate proteins eluted from the NeutrAvidin beads, with a very small amount of Cy5-RA-antibody (Fig. 5A'). Quantification of Cy5 fluorescence from antibody shows that, on an average 98.4% antibodies present in the eluate are removed following this Biotin-CDM cleanup procedure.

An important aspect of the reversibility of Biotin-CDM is that it releases proteins without the Biotin tag, which is essential for 2DE gel analysis. The NeutrAvidin eluate, which

contained Cy3- HeLa protein and Cy5-antibodies, was analyzed by 2D DIGE (Figs. 5B–D). Fig. 5C shows a wide variety of HeLa proteins were captured by the RA-anti-serum:Protein-A beads and the NeutrAvidin beads. Automated image analysis (DeCyder) of the cy3 gel image showed that there are 88 detected protein spots. These protein spots covered a wide range of molecular weights, isoelectric points (pI) and abundances. A few spots appeared to coincide with fluorescent spots in the Cy5 image of the DIGE gel (Fig. 5C) and the overlaid Cy3: Cy5 image (Fig. 5D). The vast majority of the protein spots were Cy3-labeled HeLa target proteins, demonstrating that antibodies were successfully removed and will no longer interfere with 2DE separation or mass spectrometric identification. There were few common Cy3- and Cy5-protein spots that appeared to be present in both the Cy-3 HeLa lysate and Cy5-RA-anti-serum. We speculate that these may be abundant serum proteins that are present in the RA-anti-serum and may be serum contaminants from the HeLa lysate. Mass spectrometric identification of these proteins and further experimentation will be required to determine the source of these background proteins.

3.6 Conclusions

These data demonstrate that the reagent Biotin-CDM is capable of tagging proteins from whole cell lysates to be used as targets for IP and co-IP experiments. The Biotin-CDM tag allows for a secondary isolation step whereby highly abundant antibodies can be separated from the target protein pool. Thus providing a general means to cleaning up IP and co-IP products for further proteomics analysis. The key benefit from this procedure are: (1) efficient removal of interfering antibodies from the IP target pool, (2) release of the target proteins without the complication of carrying a variable number of biotin moieties, and (3) this method provides a means to produce standard preparations of target lysates that do not require antibody-to-antibody optimization for IP experiments. The approach we describe is a combination of immunoprecipitation and 2D-DIGE that was not feasible before. Immunoprecipitation enriches low abundant proteins and thus can overcome the current limitation of SERPA in identifying low abundant protein spots. 2D-DIGE was invented to solve the gel-to-gel variability seen in 2DE experiments, the same limitation that is currently faced by SERPA. We hope this novel approach provides a valuable tool for IP, coIP based proteomics and autoantigen discovery.

Acknowledgments

We would like to thank Malachi Blundon, Anna Grace Pyzel, Danielle Schlesinger and Dr. Fred Lanni for helpful discussions on this work. We thank Dr. Tina H. Lee for allowing us to use their cell culture facility. B.S. and M.P.B. were supported by NIH TCNP grant U54GM103529. R.A. D.C. T.M. and L.T. were supported by an HHMI Undergraduate Science Education award.

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Figure 1.

Biotin-CDM Scheme.

(A) Diagrams the reversible binding reaction of Biotin-CDM with primary amines on the surface of proteins.

(B) Biotin-CDM tagged target proteins are shown as the letter 'T' with a bound Biotin, represented by two fused pentamer rings. Protein-A is shown as the letter 'A'. Antibody is shown as the letter 'Y'. Neutravidin is shown as the letter 'N'. The flowchart depicts the elution of biotinylated target proteins from antibody beads followed by antibody removal using the reversibility of Biotin-CDM.

Figure 2.

Biotin-CDM Synthesis.

Shown here is the synthetic scheme for Biotin-CDM where two molar equivalents of Biotin-2p-NH₂ hydrochloride **1** are reacted with one molar equivalent of CDM-NHS ester **2**. This reaction produces an intermediate compound that has Biotin-2p-amine bound to the maleic anhydride ring. This linkage is reversed in acid to produce Biotin-CDM.

Figure 3.

Optimizing Biotin-CDM Labeling.

(A) SDS-PAGE gel at MW 66 KD, shows the titration of increasing Biotin-CDM to BSA labeling ratio and its impact on binding of Biotinylated-BSA to NeutrAvidin beads.

(B) SDS-PAGE gel at MW 66 KD, shows the titration of increasing Biotin-CDM tagged BSA relative to NeutrAvidin beads.

(C) SDS-PAGE gel at MW 40 KD, Shows the efficacy of Biotin-CDM tagged ADH release from NeutrAvidin beads.

(D) Shows a 2D-DIGE of whole HeLa cell lysate labeled with Cy3 and whole HeLa cell lysate labeled with Cy5 and Biotin-CDM, which was bound to NeutrAvidin beads and then eluted.

(E) Shows the linear correlation between spot volumes of Cy3 and Cy5 channels from panel D. (Spot identification and quantification done using DeCyder)

Figure 4.

Biotin-CDM Capture of a Known Antigen.

(A- “normal” contrast, and A’ - “high” contrast) images of an SDS-PAGE gel containing samples Cy5-anti-p115 antibody and Cy3-tagged HeLa lysate in a control IP experiment without Biotin-CDM.

(B- “normal” contrast, and B’ - “high” contrast) images of an SDS-PAGE gel containing samples Cy5-anti-p115 antibody and Cy3-and Biotin-CDM tagged HeLa lysate in an IP experiment. B’’ Shows a magnified and contrast enhanced view of the 70–130 kD mass range of the NeutrAvidin eluate. The arrowheads indicate the putative p115 band.

C- Shows a western blot of whole HeLa cell lysate probed using rabbit anti-P115 primary and goat anti-rabbit secondary antibodies. The two bands seen are compared to the two bands seen in B''

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Figure 5.
 Biotin-CDM Capture of Unknown Serum Antigens.
 (A – “normal” contrast, and A’ – “high” contrast) images of an SDS-PAGE gel containing samples Cy5-RA-anti-serum and Cy3 and Biotin-CDM tagged HeLa lysate in an IP experiment.
 (B) Cy3 image of a 2D DIGE gel containing Cy5-RA-anti-serum and Cy3, Biotin-CDM-NeutrAvidin eluate.
 (B’) Cy5 image of a 2D DIGE gel containing Cy5-RA-anti-serum and Cy3, Biotin-CDM-NeutrAvidin eluate.

(C) Overlay of the C3 and Cy5 images of a 2D DIGE gel containing Cy5-RA-anti-serum and Cy3, Biotin-CDM-NeutrAvidin eluate.

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