

Articles

Identification of Components of Protein Complexes Using a Fluorescent Photo-Cross-Linker and Mass Spectrometry

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This study describes a novel method for improving the specific recognition, detection, and identification of proteins involved in multiprotein complexes. The method is based on a combination of coimmunoprecipitation, chemical cross-linking, and specific fluorescent tagging of protein components in close association with one another. Specific fluorescent tagging of the protein complex components was achieved using the cleavable, fluorescent cross-linker sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido) ethyl-1,3'-dithiopropionate (SAED). Following dissociation and separation by SDS-PAGE, the fluorescently tagged proteins are then visualized by UV illumination, excised, and, following in-gel digestion, identified by mass spectrometry. In this study, a complex of the HIV-envelope protein gp120 and its cellular receptor CD4 was used as a model system. The sensitivity of detection of fluorescent SAED-labeled proteins in SDS gels, and the sensitivity of the mass spectrometric identification of fluorescent proteins after in-gel digestion, is in the range of a few hundred femtomoles of protein. This sensitivity is comparable to that achieved with silver-staining techniques, but fluorescence detection is protein independent and no background interference occurs. Furthermore, fluorescence labeling is significantly more compatible with mass spectrometric identification of proteins than is silver staining. The first application of this strategy was in the investigation of the mechanism of spermiation, the process by which mature spermatids separate from Sertoli cells. For the coimmunoprecipitation experiment, an antibody against paxillin, a protein involved in spermatid-Sertoli cell junctional complexes, was used. More components of the paxillin protein complex were visible by fluorescence detection of SAED-labeled proteins than were visible on comparable silver-stained gels. Mass spectrometric analysis of the fluorescently labeled proteins identified integrin α_6 precursor as a protein associated in a complex with paxillin. The identification of integrin α_6 precursor was confirmed by Western blot analysis and verifies the applicability of this

novel approach for identifying proteins involved in protein complexes.

Multiprotein complexes are involved in numerous physiological and pathogenic processes. Isolation and identification of components of multiprotein complexes and their interaction mechanisms provide information crucial to understanding these processes and can be used to develop therapies and specific drugs to overcome diseases. Recently, several new methods to identify components of multiprotein complexes using mass spectrometry in conjunction with database searching to analyze biochemically purified complexes after SDS-PAGE separation and in-gel digestion have been described.¹ Using these approaches, numerous complexes have been identified, including the yeast nuclear pore complex and the human spliceosome.^{2,3}

The most commonly used method for the mass spectrometric identification of SDS-PAGE-separated proteins is "peptide map fingerprinting", which relies on the accurate mass measurement of peptides obtained by specific enzymatic cleavage of proteins.^{4–6} Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is commonly used for peptide mapping-based methodologies. Less sensitive than MALDI-MS, a complementary mass spectrometric approach based on electrospray tandem mass spectrometric (ESI-MS/MS) analysis has been developed which provides partial amino acid sequence information.^{7,8} Low protein concentrations, whether due to naturally low abundance or

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unavoidable loss during purification, makes MALDI-MS the first choice for identifying components of multiprotein complexes.

A common and widely used biochemical procedure to purify complexes is coimmunoprecipitation, where an antibody to immunoaffinity purify a protein of interest along with any interacting proteins from a mixture, such as cell extract, is used. One drawback of the combined approach of coimmunoprecipitation and SDS-PAGE for identifying components of multiprotein complexes is the inability to unambiguously distinguish between bands representing proteins that are part of the protein complex and those that are the result of nonspecific binding to the beads. This is often accomplished through the use of numerous controls and time-consuming, complex experiments such as GFP-labeled protein expression. Novel analytical methods providing more knowledge about the specificity of the bound protein are, therefore, of major interest.

Silver staining is typically used for detection of SDS-PAGE-separated proteins at trace levels. One limitation of this method is that the sensitivity of silver staining is protein dependent, an effect that becomes more important with decreasing protein levels. Furthermore, the gel itself may become stained, making it difficult to distinguish proteins from background staining. Finally, despite improvements, silver staining often creates difficulties during in-gel digestion leading to a loss in sensitivity in the mass spectrometric identification of proteins.⁹

Recently, fluorescent stains such as SYPRO Red and SYPRO Ruby have been used to enhance the detectability of gel-separated proteins. These fluorescent stains have been found not to interfere with the subsequent mass spectrometric identification of these proteins.^{10,11} In the method described here, components of a multiprotein complex are fluorescently tagged before they are separated on a gel, instead of fluorescently staining all proteins on the gel after the separation. Since only the proteins from the complex are fluorescently tagged, their detectability can be significantly enhanced. These proteins of interest can then be identified by in-gel digestion and mass spectrometric analysis.

The method used in this paper is based on a combination of (1) coimmunoprecipitation of the noncovalent protein complex, (2) specific fluorescent labeling of the protein components linked to each other in the complex by cross-linking with a cleavable cross-linker, (3) cleavage of the cross-linker by reduction, (4) SDS-PAGE with fluorescence detection of the labeled complex members, and (5) subsequent identification of fluorescent proteins by MALDI-MS (Figure 1).

For specific fluorescent labeling, the complex is covalently bound using the cleavable, fluorescent cross-linker sulfo-succinimido-2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3'-dithiopropionate (SAED) (Figure 2). SAED possesses two different reactive groups that allow for sequential conjugation of proteins, minimizing undesirable polymerization or self-conjugation, and targets a fluorophore to the vicinity of binding sites of interacting proteins via a coumarin group.¹² Upon cleavage of the central disulfide

spacer of the SAED, the fluorescently labeled proteins in the complex are released.

The first application of this strategy was used to investigate spermiation, the process by which late spermatids separate from Sertoli cells, allowing release of spermatozoa into the lumen of the seminiferous tubule.¹³ A number of toxicants and pharmaceuticals disrupt this process, and consequently, the inhibition of spermiation is of particular interest. Unfortunately, the mechanisms involved in spermiation are poorly understood. It has been hypothesized that a transient cytoskeletal-transmembrane protein signal transduction complex is formed that helps control the process of sperm release.^{14,15} Presumably, this complex would also signal the Sertoli cell that release of spermatozoa has occurred. By immunohistochemistry, a number of proteins (vinculin, tubulin, paxillin, etc.) can be detected which appear to be associated with the spermatid-Sertoli junction.¹⁵ Because of its adluminal distribution and localization to the spermatid-Sertoli cell junction, antibodies against paxillin were used for the coimmunoprecipitation experiments.¹⁶

MATERIALS AND METHODS

Materials. Recombinant soluble CD4 (sCD4) from R. Sweet (SmithKline Beecham) was obtained through the AIDS Research Reagent Program, NIAID, NIH. sCD4 comprises amino acids 1–369 of the mature CD4 protein.¹⁷ HIV-gp120 was purchased from Austral Biologicals (San Ramon, CA). 'SAED was purchased from Pierce (Rockford, IL). The α -cyano-4-hydroxycinnamic acid (CHCA) was from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized from methanol before use as a MALDI matrix.

Animals. Adult male Sprague-Dawley rats (CrI:CD, 70–180 days of age) were purchased from Charles River Laboratories (Raleigh, NC) and were acclimated to the NIEHS animal facility. All the animals were housed in polycarbonate cages with 12:12-h light/dark cycles, 50 \pm 10% humidity, and ambient temperature of 20 \pm 1 °C and were given NIH-31 diet and water ad libitum, all in accordance with the *NIEHS Guidelines for the Humane Care and Use of Animals in Research*. Animals were killed by asphyxiation with CO₂, the testes were removed, and lysates of stage VIII seminiferous tubules were prepared as previously described.¹⁴

Mass Spectrometry. The MALDI analyses for the model experiment were performed on a DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) in the positive ion reflector mode with delayed extraction using an accelerating voltage of 25 kV. For the analyses of the in-gel-digested proteins after coimmunoprecipitation with paxillin, a Voyager-RP time-of-flight mass spectrometer (Applied Biosystems) was used in the positive ion linear mode. The accelerating voltage was set at 30 kV in positive ion linear mode. Both MALDI mass spectrometers were equipped with a nitrogen laser (λ = 337 nm). For the sample preparation, a saturated solution of CHCA in ethanol/water/formic acid (45:45:10), freshly prepared, was used

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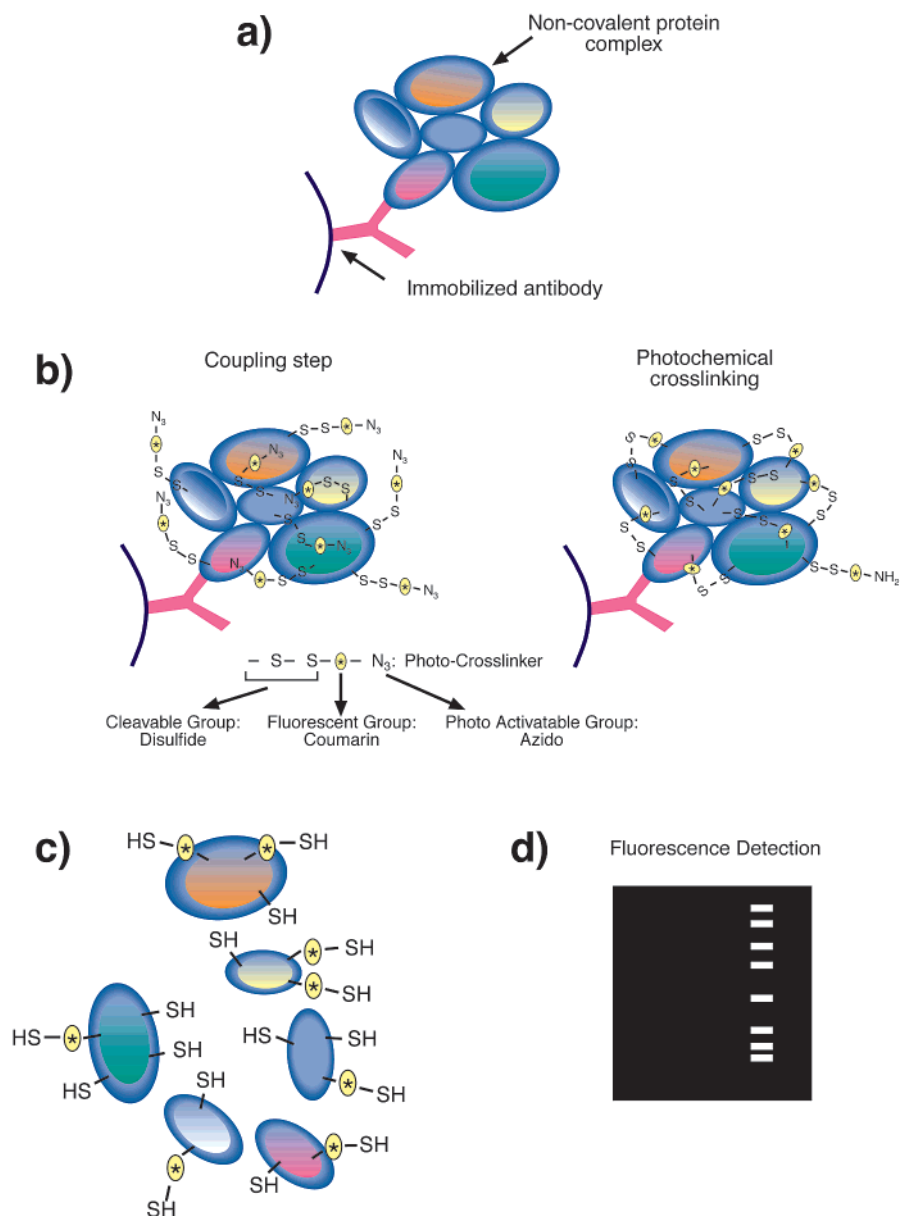


Figure 1. Analytical strategy for specific labeling and detection of components of protein complexes using a fluorescent photo-cross-linker. (a) Coimmunoprecipitation of the noncovalent protein complex. (b) Fluorescent labeling of the complex using a cross-linker possessing a cleavable disulfide group, a fluorescent coumarin group, and a photoactivatable azido group. The cross-linker is covalently incorporated into the complex by chemically reacting with free amino groups, mainly from lysine residues, in proteins. The proteins of the complex are subsequently cross-linked by photochemical reaction of the cross-linker's azido group. (c) Cleavages of the disulfide group in the cross-linker by reduction releases the fluorescently labeled proteins. It also removes the fluorescent group from proteins that are not cross-linked. This enhances the labeling specificity of complex components. (d) The fluorescent-labeled components of the protein complex are separated by SDS-PAGE and detected by fluorescence imaging.

as the MALDI matrix. Samples (0.5 μ L) were spotted on the target followed by 0.5 μ L of matrix solution and were allowed to dry at room temperature.

Database Searching. The peptide mass data was searched against the NCBI database using the programs Prospector and Prowl (Internet addresses: <http://prospector.ucsf.edu/> and <http://prowl.rockefeller.edu/>).

Cross-Linking. (1) HIV-gp120/sCD4 Complex. The cross-linking of the HIV-gp120/sCD4 complex was described previously.¹⁸ Briefly, after buffer exchange of the sCD4 solution against

20 mM phosphate buffer, pH 7.5, the noncovalent complex was formed by incubation of HIV-gp120 and sCD4 at a molar ratio of 1:1.4 (HIV-gp120/sCD4) in 20 mM phosphate buffer, pH 7.5, at room temperature under rotation for 1 h. The complex was allowed to react in the dark with a 10-fold molar excess of freshly prepared SAED. The reaction was quenched by adding a 30-fold molar excess of lysine (Sigma Chemical Co., St. Louis, MO), and the derivatized complex was separated from free reagent by gel filtration through spin chromatography columns (Bio-Rad, Hercules, CA) filled with porous polyacrylamide (Bio-Gel P-2 from Bio-Rad). Photoactivation was carried out for 15 min by exposing

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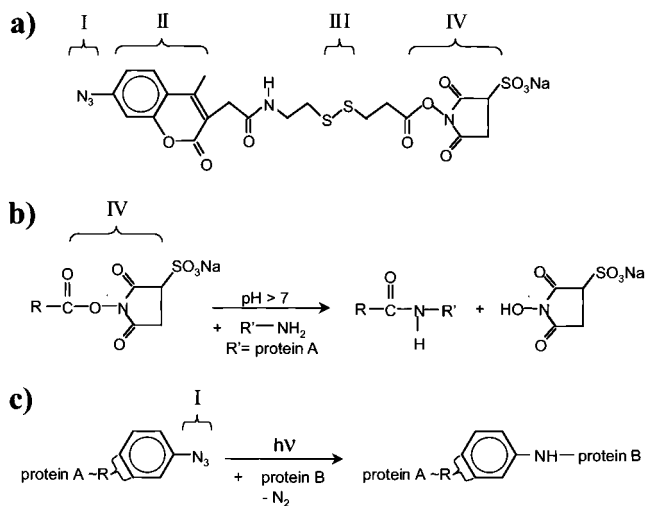


Figure 2. Scheme for protein-protein cross-linking using SAED. (a) Chemical name and structure of SAED. SAED is a heterobifunctional (I, photoactivatable azido group; IV, acylating NHS ester), cleavable (III, disulfide group), fluorescent (II, coumarin group) cross-linker. Cross-linking is performed by sequential reactions of (b) the NHS ester of the cross-linker with an amino group followed by (c) azidophenyl photolysis, which leads to the covalent conjugation of interacting proteins.

the sample to a black-ray, long-wave, 100-W ultraviolet lamp (Ultra Violet Products Inc., San Gabriel, CA). $2\times$ Tris-glycine SDS samples were diluted 1:1 in nonreducing sample buffer (Invitrogen-Novex, Carlsbad, CA), heated for 5 min at 56°C , and incubated for 30 min at 22°C before electrophoresis.

(2) Paxillin Complex. The paxillin coimmunoprecipitation was performed at 4°C in 20 mM phosphate buffer, pH 7.5, using precleared lysates¹⁹ from stage VIII seminiferous tubules prepared as previously described.¹⁴ The lysates were precleared by incubating with protein G agarose (Invitrogen Life Technologies, Carlsbad, CA) and $5\text{ }\mu\text{g}$ of normal mouse serum (Sigma) under rotation. The protein G agarose was removed by centrifugation, and the supernatant was combined with a fresh volume of r protein G agarose. Following additional incubation, the r protein G agarose was again removed by centrifugation and the supernatant (precleared lysate) was stored on ice until use. All procedures were performed at 4°C unless otherwise noted.

A $5\text{-}\mu\text{g}$ sample of monoclonal anti-paxillin antiserum (BD Transduction Laboratories, Lexington, KY), $5\text{ }\mu\text{g}$ of monoclonal anti-paxillin antiserum (Zymed Laboratories, San Francisco, CA), and $500\text{ }\mu\text{g}$ of precleared stage VIII seminiferous tubule lysate were combined in $2\times$ coimmunoprecipitation buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 50 mM NaF, and 0.5 mM Na_3VO_4) and co-incubated for 1 h with gentle end-over-end rotation. A $10\text{-}\mu\text{g}$ sample of polyclonal anti-mouse IgG (Sigma) was added to the immune complexes, and incubation was continued for an additional 30 min. The immunocomplexes were captured by the addition of $10\text{ }\mu\text{L}$ of prewashed r protein G agarose. Following a 30-min incubation, the complexes were pelleted by centrifugation, and the pellet was washed three times with $1\times$ coimmunoprecipitation buffer and three times with 20 mM phosphate buffer, by suspension/centrifugation.

After the final wash, the pellet was resuspended in $20\text{ }\mu\text{L}$ of phosphate buffer and incubated with SAED as described above.

The cross-linking reaction was quenched with lysine, the complexes were pelleted by centrifugation, and the excess SAED was removed by suspension/centrifugation three times with 20 mM phosphate buffer. After the final wash, the pellets were resuspended in $100\text{ }\mu\text{L}$ of 20 mM phosphate buffer and photoactivation was performed as described above. The pellet was collected and resuspended in $10\text{ }\mu\text{L}$ of 0.1 M SDS/0.1 M β -mercaptoethanol and was incubated at 60°C for 45 min to reduce the cross-linker. The samples were diluted 1:1 with $2\times$ Tris-glycine SDS sample buffer (Invitrogen-Novex) and then stored on ice until electrophoresis.

Gel Electrophoresis and Imaging Analysis. SDS-polyacrylamide gel electrophoresis was performed using precast 1-mm-thick 10% Tris-glycine gels (Invitrogen-Novex) according to the manufacturer's instructions. The proteins were separated at 125 V constant voltage until the bromophenol blue dye front reached the bottom of the gel and then postfixed for 20 min in 10% acetic acid (Mallinckrodt Baker, Paris, KY)/25% 2-propanol (J.T. Baker, Phillipsburg, NJ). Silver staining was performed using a kit from Bio-Rad according to the manufacturer's instruction. To determine the amount of protein in separated bands, a Coomassie staining was performed with 0.006% Coomassie Brilliant Blue R-250 (Bio-Rad) in 10% acetic acid for several hours. Destaining was performed with 10% acetic acid. Quantitation of the protein bands, as well as visualization and documentation of the fluorescently labeled proteins, was performed using a Kodak ImageStation 440CF (Eastman Kodak, Rochester, NY) with either transmitted or UV epi-illumination and a 523- or 398/480-nm band-pass filter, respectively.

In-Gel Digestion. Protein bands were excised from the SDS gel and cut into $\sim 1\text{ mm}^3$ pieces. In-gel digestion was performed either manually or automatically using a Progest automated digestion apparatus (Genomic Solutions, Ann Arbor, MI), according to a procedure that was previously described.²⁰ Briefly, gel pieces were desalted, dehydrated, and reswelled in 25 mM ammonium bicarbonate containing $10\text{ }\mu\text{g/mL}$ porcine trypsin (Promega, Madison, WI). Digestion was performed at 37°C for 4 h using the Progest or for 18 h using the manual mode. After digestion was complete, samples were lyophilized and reconstituted for mass spectrometric analysis in $5\text{ }\mu\text{L}$ of 50% acetonitrile/0.1% formic acid.

Western Blot Analysis. Western blots were performed according to routine methods. Briefly, coimmunoprecipitates were diluted 1:1 with $2\times$ Tris-glycine SDS sample buffer (Invitrogen-Novex), loaded onto a 4–12% Tris-glycine polyacrylamide gel (Invitrogen-Novex), and separated, under reducing conditions, according to the method of Laemmli.²¹ Resolved proteins were transferred to $0.2\text{-}\mu\text{m}$ nitrocellulose membrane using Towbin's transfer buffer and blocked with Superblock (Pierce). Primary antisera against paxillin (Pharmingen-Transduction Laboratories) and integrin α_6 (CD49f, Upstate Biotechnology, Lake Placid, NY) were used at dilutions of 1:10 000 and 1:250, respectively. The antigen(s) of interest was(were) detected using SuperSignal West Pico chemiluminescent substrate (Pierce), and the images were acquired on a Kodak ImageStation 440CF (Eastman Kodak). Biotinylated markers (Bio-Rad) and SeeBlue Plus2 prestained standards (Invitrogen-Novex) were separated in conjunction with

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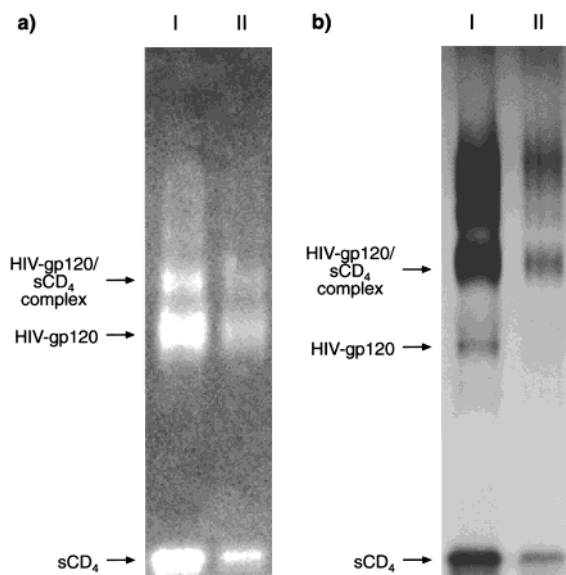


Figure 3. SDS-PAGE of the HIV-gp120/sCD4 complex following cross-linking with SAED and detection by (a) fluorescence imaging and (b) silver staining. The protein amounts for sCD4, HIV-gp120, and the HIV-gp120/sCD4 complex in lane I are 800, 500, and 160 fmol, and in lane II, 400, 250, and 80 fmol, respectively.

the samples to allow for accurate determination of molecular weight and efficiency of transfer, respectively. A 10- μ g aliquot of total protein from lysates of whole rat testis, human endothelial, and A431 cells was included on each gel as a positive control to verify the specificity of the antisera and confirm the presence of the protein in rat testis. All Western blots were performed at least three times.

RESULTS AND DISCUSSION

Model System. To determine the sensitivity of fluorescent photo-cross-linking combined with mass spectrometry for detection of fluorescent protein bands and mass spectrometric identification of in-gel-digested proteins, the complex of the human immunodeficiency virus glycoprotein 120 (HIV-gp120) with its cellular receptor CD4 was chosen as a model. As recently shown, the HIV-gp120/sCD4 complex occurs as a one-to-one complex in solution with complexes of a higher order of stoichiometry not being detected.¹⁸ Identical results were obtained by cross-linking the HIV-gp120/sCD4 complex using SAED with subsequent analysis by MALDI-MS and SDS-PAGE after Coomassie staining.¹⁸ To determine the detection sensitivity of SAED-derivatized/SAED-cross-linked proteins by fluorescence imaging of polyacrylamide gels and to compare those to the detection sensitivity obtained from silver staining, the HIV-gp120/sCD4 complex was cross-linked with SAED and applied to a polyacrylamide gel at two different protein amounts (Figure 3). For quantification of the amounts of protein applied, densitometric analyses of protein bands after Coomassie staining, using HIV-gp120 and sCD4 as standards, were performed in a separate experiment.

Since the reaction yield for the cross-linking of the HIV-gp120/sCD4 complex with SAED is known, the amount of protein in the complex could be determined.¹⁸ The proteins sCD4, HIV-gp120, and their binary complex were found to present at 800, 500, 160, and 400, 250, and 80 fmol in lane I and lane II of Figure 3,

respectively. The fluorescence imaging of the gel showed three bands in each of the two lanes, which corresponded to the non-cross-linked, SAED-derivatized proteins sCD4 and HIV-gp120 and the SAED-cross-linked HIV-gp120/sCD4 complex (Figure 3a). The fluorescence intensity of the protein bands reflected the amount of protein present on the gel. Using silver staining, the protein bands for sCD4 and the HIV-gp120/sCD4 complex were detected in both lanes (Figure 3b, lanes I and II); however, the protein band corresponding to HIV-gp120 could only be detected in lane I (500 fmol of HIV-gp120 applied to the gel). Furthermore, silver staining resulted in intense staining of broad, smeared bands, in the upper molecular weight range at both lanes, neither of which was detected by fluorescence imaging or by Coomassie staining.¹⁸ These smeared bands may correspond to contaminants of HIV-gp120, revealed by silver staining of HIV-gp120 in a separate experiment. These bands also could be, albeit to a lesser extent, higher orders of HIV-gp120/sCD4 complexes, background staining, or a combination of all of these possibilities.

Regarding compatibility of fluorescence imaging versus silver staining with mass spectrometric analysis of the in-gel-digested proteins, the sCD4 bands in lane II from Figure 3a and b were excised and digested with trypsin. For the digestion, an automated digester was used to ensure identical treatment of both samples. The MALDI-MS spectrum of in-gel-digested sCD4 after fluorescence imaging contained 11 ion signals that were identified as sCD4 peptides using protein database searching, corresponding to 25% amino acid sequence coverage (Figure 4a). This search query identified CD4 with a confidence level above the 99th percentile (Z-score, 2.36), reflecting a probability of a false positive protein identification of less than 1%.²² Furthermore, these results showed that, despite modification of the protein, the in-gel digestion is still sufficient for an unambiguous protein identification, since only a limited number of peptides carried the cross-linker.¹⁸ In the MS analysis of the silver-stained sCD4, the MALDI-MS showed only two ion signals that corresponded to sCD4, and these were barely detectable above noise level (Figure 4b). A protein database search also yielded sCD4, but at a low confidence level, and the identification was therefore ambiguous (Z-score, 0.13).

Proof of Principle Experiment. We applied our method of mass spectrometric analysis of proteins detected by fluorescence labeling and SDS-PAGE to the identification of novel proteins involved in the process of spermiogenesis. An antibody against paxillin, which is known to be associated with the spermatid-Sertoli cell junction, was used to coimmunoprecipitate other proteins in this complex from a rat testis lysate. The coimmunoprecipitated proteins were cross-linked with SAED, reduced, and separated by SDS-PAGE, and the gel was analyzed by fluorescence imaging followed by silver staining (Figure 5). The silver staining showed three protein bands (Figure 5a, lane II). In addition to these bands, the fluorescence imaging revealed three more protein bands (Figure 5b, lane II), two in the lower molecular weight range (\sim 20 000, bands 1 and 2) and one band at \sim 120 000 (band 6). It should be noted that protein band 6 is not distinguishable from the intense background staining at this molecular weight range in the silver-stained gel but is clearly visible by fluorescence. The two lower molecular weight proteins correspond to the light chains of the antibody as shown by SDS-PAGE analysis of the

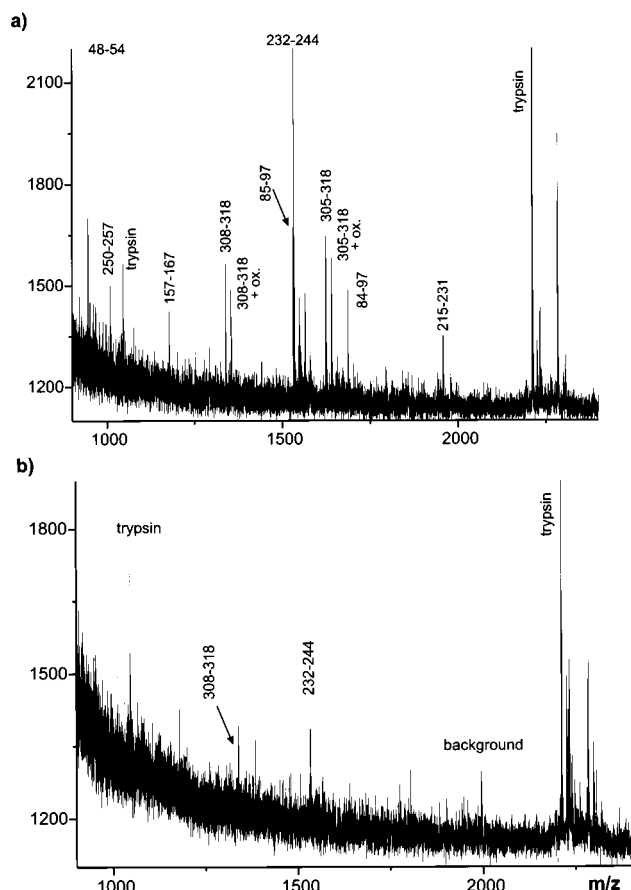


Figure 4. MALDI-MS analysis of the in-gel-digested sCD4 band in the SDS-PAGE gel after (a) fluorescence imaging and (b) silver staining (see Figure 3a,b, lanes II). The ion signals corresponding to sCD4 tryptic peptides are annotated according to their positions in the amino acid sequence. The tryptic autoproteolytic peptides are annotated as trypsin.

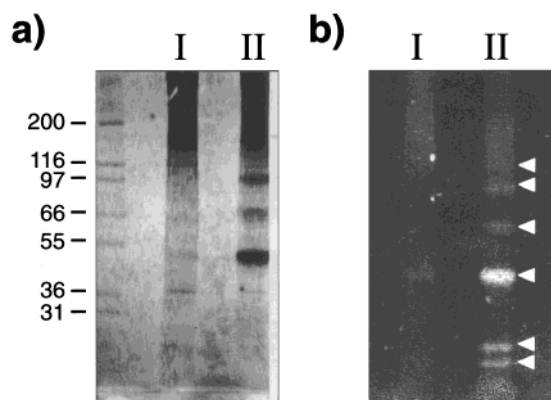


Figure 5. SDS-PAGE of coimmunoprecipitated proteins from testis lysate after (a) silver staining and (b) fluorescence imaging. The coimmunoprecipitation was performed using normal serum as a control (lane I) and an anti-paxillin antibody.

antibody (data not shown). This experiment also showed that protein band 3 belongs to the antibody (heavy chain) as well. The protein band 3 is also visible in the control experiment in which the immunoprecipitation was performed with normal serum (Figure 5 a,b, lanes I).

Because the gel was already silver stained, protein bands 4 and 5 and the area where protein band 6 appeared by fluorescence

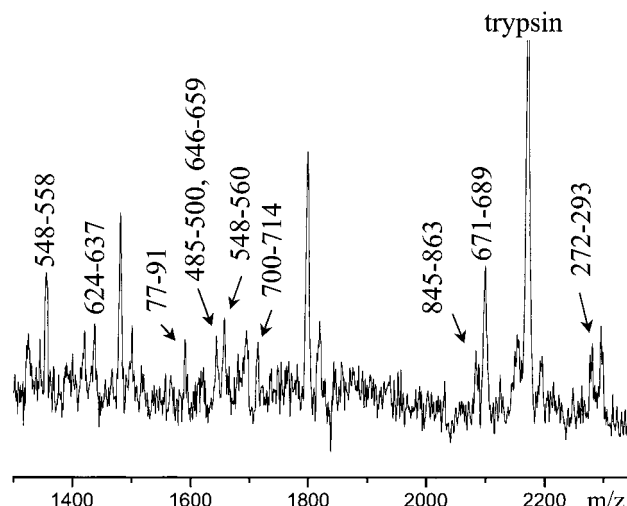


Figure 6. MALDI-MS analysis of the in-gel-digested protein band 5 in the SDS-PAGE gel after silver staining (see Figure 5a, lane II). The ion signals corresponding to the tryptic peptides of integrin α_6 precursor are annotated by their positions in the amino acid sequence.

imaging were excised and in-gel digested with trypsin. MALDI-MS analysis of in-gel-digested protein band 4 combined with database searching identified this protein as paxillin against which the antibody, used in this immunoprecipitation experiment, was raised (data not shown). This result was also confirmed by Western blot analysis (data not shown).

The MALDI-MS spectra obtained after in-gel digestion of protein bands 5 (Figure 6) and 6 were very similar, but the intensities of the ion signals from band 6 were lower (data not shown). A database query for these proteins resulted in the identification of integrin α_6 precursor from mouse with a MW of 119 600. This molecular weight is in very good agreement with the gel-determined molecular weight of protein band 6, but is $\sim 25\,000$ higher than that of the gel-determined molecular weight of protein band 5. This suggests that band 5 may be a splice variant of integrin α_6 or a truncated or posttranslationally modified form of the protein.

To confirm the mass spectrometric identification of protein bands 5 and 6 and demonstrate that integrin α_6 is associated with paxillin and involved in the spermiation process, Western blot analysis with an anti-integrin α_6 antibody was performed on proteins precipitated with the anti-paxillin antibody (Figure 7). The coimmunoprecipitations were performed just before and immediately after spermiation. Western blot analysis demonstrated that integrin α_6 appears in two forms with apparent MWs of 120 000 and 95 000–100 000, which correspond to the apparent molecular weights of protein bands 5 and 6 (Figure 7, lanes IV and V). When the immunoprecipitation was performed with normal serum, no integrin α_6 was detected in the negative control (Figure 7, lane II, III).

Western blotting revealed that integrin α_6 is associated with paxillin and that this complex is present both before and after sperm release. This finding was recently confirmed in our laboratory; in the absence of cross-linking, integrin α_6 was found as part of a complex formed with paxillin, β_1 integrin, and several other proteins.¹⁴ Moreover, other investigators have localized $\alpha_6\beta_1$ integrins to defined junctional structures thought to mediate spermatid–Sertoli adhesions.^{16,23,24}

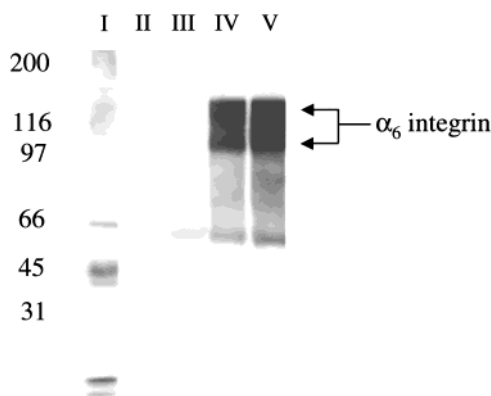


Figure 7. Western blot analysis of coimmunoprecipitated proteins from testis lysate using an anti-(integrin α_6 precursor) antibody. Lane I: biotinylated molecular weight markers. The coimmunoprecipitation was performed using normal serum as a negative control (lanes II + III) and an anti-paxillin antibody (lanes IV and V). The coimmunoprecipitation was carried out directly before spermiation (lanes II + IV) and after spermiation (lanes III and V).

CONCLUSION

The combination of specific fluorescence labeling using a photo-cross-linker and mass spectrometric analysis of proteins enzymatically in-gel digested, followed by protein database searching, described here, is a powerful approach for the identification of components of protein complexes because of the distinct advantages it offers over traditional techniques in functional proteome analysis. Fluorescent labeling can improve the detectability of proteins involved in multiprotein complexes, since the labeling occurs by fluorescence transfer from one protein to another if they are in close proximity. Using a photoactivatable cross-linker, undesirable self-labeling is minimized, and in contrast to the cross-linker directed to functional amino acid residues, no specific amino acid residue is required to be present in close proximity to the incorporated cross-linker.

In our hands, the absolute sensitivity of fluorescence imaging was comparable to that of silver staining. Fluorescence imaging, however, is protein independent, causes no background interference, and has a higher dynamic range, and the intensity of the fluorescence correlates with the amount of protein present. Furthermore, fluorescent labeling can enhance the specificity of

recognition of proteins involved in the complex. In addition, cross-linking might increase the stability of the complex, making the analysis of fragile complexes more feasible. This is especially apparent if the cross-linking has been performed prior to the coimmunoprecipitation. Cross-linking prior to coimmunoprecipitation also allows the researcher to distinguish between specifically and nonspecifically bound complexes. The complexes are first separated by SDS-PAGE (low-percentage acrylamide gels) without any reducing reagent, to keep the cross-linked complex intact. The intact complexes are next in-gel reduced and alkylated to improve the enzyme accessibility, and finally in-gel digested with trypsin. A specifically bound complex must contain the protein against which the immunoprecipitation was performed. Thus, mass spectrometric identification of this protein in the gel-separated complex allows one to distinguish between specific and nonspecifically bound complexes. Another important advantage of this novel approach is that, compared to silver staining, fluorescent labeling is far more compatible with mass spectrometric analysis of in-gel-digested proteins. This feature ultimately results in a higher overall sensitivity for identification of components of protein complexes than with traditional techniques.

This combined approach of fluorescent photo-cross-linking and mass spectrometric analysis also shows great promise for the characterization of interaction sites in multiprotein complexes, since the use of SAED leads to the attachment of fluorophores in the vicinity of the binding sites between interacting proteins. With this technique, the coimmunoprecipitated and cross-linked complex is enzymatically digested while bound to affinity beads and the digestion mixture is separated by liquid chromatography equipped with a fluorescence detector.²⁵ The isolated, fluorescently labeled, cross-linked peptides are analyzed on- or off-line by liquid chromatography and tandem mass spectrometry, which allows the identification of the interacting proteins and reveals the binding sites. Additional improvement in the mass spectrometric detection of cross-linked peptides can be achieved by using a stable isotope-labeled fluorescent photo-cross-linker.²⁶ Thus, mass spectrometric results can be used to determine the quaternary structure of the multiprotein complexes, providing important insights into their biological function.

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