Identification of Keratinocyte-specific Markers Using Phage Display and Mass Spectrometry* 

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Specific molecular markers for various normal and pathogenic cell states and cell types provide knowledge of basic biological systems and have a direct application in targeted therapy. We describe a proteomic method based on the combination of new and improved phage display antibody technologies and mass spectrometry that allows identification of cell type-specific protein markers. The most important features of the method are (i) reduction of experimental noise originating from background binding of phage particles and (ii) isolation of affinity binders after a single round of selection, which assures a high diversity of binders. The method demonstrates, for the first time, the ability to detect, identify, and analyze both secreted and membrane-associated extracellular proteins as well as a variety of different cellular structures including proteins and carbohydrates. The optimized phage display method was applied to analysis of human skin keratinocytes resulting in the isolation of a panel of antibodies. Fourteen of these antibodies were further characterized, half of which predominantly recognized keratinocytes in a screen of a range of different cell types. Three cognate keratinocyte antigens were subsequently identified by mass spectrometry as laminin-5, plectin, and fibronectin. The combination of phage display technology with mass spectrometry for protein identification is a general and promising approach for proteomic analysis of cell surface complexity. *Molecular & Cellular Proteomics 2: 61–69, 2003.

The epidermis has very important functions such as protecting the organism against environmental hazards, e.g. microbes and stress, and keeping fluids inside the body. The main cell type found in the epidermis is the keratinocyte, which makes up more than 80% of the cells (1). Although the understanding of keratinocyte proliferation and differentiation is increasing, additional markers for single cell states are always required (2).

A variety of different methods, e.g. antibody technology (3), two-dimensional PAGE, mass spectrometry (4), DNA arrays (5), and protein arrays (6), have been used either alone or in combination to analyze protein expression. However, the majority of these methods have difficulties analyzing high molecular weight, transmembrane, and extracellular proteins as well as posttranslational modifications such as glycosylation and proteolytically processed proteins. Increased knowledge of these components is vital for understanding biological processes such as those involved in cell communication. Furthermore, an intrinsic problem of these methods is that the average cellular distribution in a cell population is analyzed, hindering the investigation of minor protein fractions with subtle localization.

Analysis of cell-specific protein expression using conventional antibody technology has utilized immunization with preparations of the cell type or tissue and concomitant analysis of antibody specificities (3). Recently phage display has played an important role in the generation of such cell type-specific antibodies and the ensuing identification of cell markers (for reviews, see Refs. 7–9). Several approaches have been reported for identification of cell markers such as selection using activated cell sorting (10, 11), selection directly on cells in suspension (12), selections directly on adherent cells (13, 14), and selection on tumor tissue sections (15). Recently another approach has been described enabling the identification of cell surface components capable of internalizing binding ligands (16).

In the present study we applied a protease-sensitive helper phage (17) in cell surface selections on cultured human keratinocytes. The protease-sensitive helper phage has previously been used successfully in the generation of phage display-derived antibodies by increasing the fraction of target-specific antibodies (18–20). The antibody repertoires used in the present study are conceptually different, namely a semisynthetic repertoire, the Griffin library,¹ and two single framework repertoires, the Tomlinson I and Tomlinson J (20). One of the most important features of the established method is that it enabled screening of monoclonal phage antibodies after a single round of selection. This ensured a high diversity of binding antibodies, some of which recognized markers specifically produced by the target. These markers were either membrane-associated or soluble. Other antibodies had

¹ www.mrc-cpe.cam.ac.uk/winter-hp.php?menu=1808.
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cross-reactivity to epitopes presented by other cell types. Three antigens were subsequently isolated, and their identities were determined using mass spectrometry. This study demonstrates the potential of applying a new helper phage in complex selections since it reduces the number of antibody-displaying phage retrieved nonspecifically. Furthermore, it exemplifies the powerful combination of mass spectrometry and phage antibody technology in analyses of cell type-specific expression profiles.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Primary human keratinocytes were isolated from mammary tissue and cultured according to Norsgaard et al. (22). Cells other than keratinocytes were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) containing 10% fetal calf serum, 1 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Furthermore, the cells were incubated at 37 °C in 5% CO2 and 99% humidity.

**Bacterial Strains and Phage-displayed Antibody Repertoires**—The amber codon suppressing Escherichia coli strain TG1 (supE hsdD5 Δ(lac-proAB)φ80 lacZΔM15) was used for amplification of phage displaying antibody fragments. The Griffin repertoire as well as the Tomlinson I and J repertoires are described elsewhere (20).

**Phage Propagation**—Antibody repertoires were rescued either with the protease-sensitive helper phage KM13 or the traditional helper phage M13K07 (Stratagene) as described in Refs. 17 and 18.

**Selection of Monoclonal Phage Antibodies**—The selections with KM13-rescued repertoires were performed on cultured keratinocytes in six-well plates (Costar). A total number of 2 × 10^10^ keratinocytes were added to each well 1 day prior to selection to obtain 80% confluence. After overnight incubation in a cell incubator (37 °C, 99% humidity, and 5% CO2) the wells were blocked with 2% MPBS (PBS supplemented with 2% (w/v) low fat milk powder) for 2 h at room temperature. Meanwhile phage antibody repertoires were separately incubated with 50:50 volume % 4% MPBS and keratinocyte medium (Invitrogen).

After blocking the wells were briefly rinsed with PBS and incubated with phage repertoire for 1.5 h at room temperature in 3 ml of 50:50 volume % 4% MPBS and keratinocyte medium. The wells were then washed six times for 5 min with PBS. Bound phage were specifically eluted using trypsin (1 mg/ml in 1 mM CaCl2, 50 mM Tris-HCl, pH 7.4). Trypsin proteolyses the Myc tag that separates the single chain Fv and protein III as well as the protease-sensitive linker in KM13-encoded protein III. A log-phase TG1 culture was infected with eluted phage and plated on TYE (10 g/liter peptone, 5 g/liter yeast extract, 8 g/liter NaCl, 15 g/liter agar) plates containing ampicillin (200 μg/ml) and glucose (1%). The selection with the Griffin repertoire was propagated for a second round of panning by rescue with helper phage KM13 as described in Refs. 18 and 23.

The control selection with the M13K07 (Stratagene)-rescued Griffin repertoire was performed using the conditions described above and in Marks et al. (23) except for elution of phage with 100 mM triethylamine and subsequent neutralization with 1 mM Tris-HCl, pH 7.5 before infection into log-phase TG1.

**Monoclonal Phage Antibody ELISA**—Monoclonal phage antibody clones were rescued overnight with KM13 for ELISA (18). Afterward cultures were cleared by centrifugation, and phage were precipitated by addition of 1:5 volume of 20% polyethylene glycol 6000, 2.5 M NaCl. Phage were pelleted by centrifugation and resuspended in 1:1 volume of PBS. ELISA was performed on the indicated cell type by culturing overnight in 96-well plates (5000 cells/well, Costar) with the appropriate medium. Cell culture medium, either conditioned or not conditioned by cell culturing, was analyzed by overnight coating in ELISA plates (MAXI-sorp™, Nunc, Roskilde, Denmark). Next wells were incubated with 2% MPBS for 2 h before addition of 5 × 10^10^ monoclonal phage in 100 μl of 2% MPBS and incubation for 1 h. After phage incubation, the wells were washed six times in PBS, and bound phage were detected with a horseradish peroxidase-conjugated mouse anti-phage antibody (Amersham Biosciences). ELISAs were developed with o-phenylenediamine tablets (Dako) according to the manufacturer’s instructions.

**Production of Soluble Antibodies**—Antibodies were cloned into the novel expression vector pKB1J to allow expression of active and soluble antibody (24). Briefly, the vector directs the production of antibodies fused to the N terminus of filamentous coat protein III domain I-III to the periplasmic space of the bacteria. Fusion to protein III domain I-III functionally rescues otherwise inactive phage display-derived antibodies.

**Analysis of Medium Obtained from Keratinocyte Cultures**—Medium was obtained from 80% confluent culture flasks after 2 days of culturing and cleared from cell material by centrifugation (3000 × g, 20 min). Conditioned medium was dialyzed in low salt buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl) and subjected to Q-Sepharose fractionation applying a gradient of NaCl. Ten microliters of each fraction were coated in ELISA wells and tested for the presence of antigen. Next antigen-containing fractions were concentrated by centrifugation in Centricron 3 (Amicon Bio separations) and subjected to gel filtration on an TSK-gel G3000 SW column (ToSoHaas) using HPLC (Biotek Instruments). Positive fractions from ion-exchange chromatography were pooled and dialyzed in acetic acid buffer (50 mM sodium acetate, pH 4.8, 50 mM NaCl) leading to protein precipitation. The resulting pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl.

**Extraction of Membrane-associated Antigens**—Confluent keratinocyte cultures were metabolically labeled overnight with a 1:1000 dilution of a Redivue PRO-MIX™ 5× cell labeling mix (Amersham Biosciences) in keratinocyte medium. After labeling, cultures were washed twice with PBS and incubated with antibody clones 12 and B3, which had been biotinylated using an ECL™ protein biotinylation module (Amersham Biosciences) according to the manufacturer’s instructions. Next antigen-antibody complexes were precipitated by incubation with PBS supplemented with 0.5% Triton X-100 before incubation with 1.7 × 10^7 streptavidin-coated Dyna™ beads (Dyna). Subsequently beads were washed, and bound protein was analyzed using SDS-PAGE.

**Protein Digestion and MALDI Mass Spectrometry Analysis**—Proteins were isolated by SDS-PAGE. Protein bands were excised and treated with dithiothreitol, S-alkylated with iodoacetamide, and then incubated with trypsin (sequencing grade, modified porcine trypsin, 12 ng/μl, Promega, Madison, WI) as described previously (25). Peptides were extracted, dried, and redissolved in 20 μl of 5% formic acid prior to mass analysis. Peptide mixtures were analyzed using a RE-FLEX MALDI reflector time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) operated in the positive ion delayed extraction mode. Peptide mixtures were desalted and concentrated using custom-made nanoscale columns (26) and deposited onto the MALDI target by elution with the matrix solution. Peptide mass spectra were mass calibrated by using trypsin autolysis peptide signals (m/z 842.51 and 2211.10) generating a mass error of less than 50 ppm.

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2 The abbreviations used are: MPBS, PBS supplemented with low fat milk powder; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TRITC, tetramethylrhodamine isothiocyanate. 

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for all peptides (27). Proteins were identified via their peptide mass maps by using the ProFound search engine (Genomic Solutions) and the Mascot search engine (Matrix Science Ltd., London, UK).

Verification of the Antigen Identity—ELISA was performed on purified recombinant laminin-5, which was a kind gift from Dr. Kaoru Miyazaki, Yokohama City University (28), and purified human fibronectin (Sigma). One microgram of purified protein was coated overnight at 4 °C in 50 mM NaHCO₃, pH 9.6. Phage-displayed antibodies D4 and 12 were incubated with the wells, and the ELISA was performed as described above for monoclonal phage ELISAs. Indirect immunofluorescence studies were performed on keratinocytes fixed and permeabilized using PBS supplemented with 1% formaldehyde and 0.1% Tween 20 for 5 min at room temperature. Residual binding to the glass surfaces of wells was blocked with 2% MPBS before incubating with ~10 μg/ml biotinylated antibody B3 in the presence of 2% MPBS. Next biotinylated antibodies were detected using a 1:400 dilution of fluorescein isothiocyanate-conjugated Extra-vi
din® (Sigma). A commercially available monoclonal mouse anti-nectin antibody (Sigma) was applied in a 1:100 dilution for colocalization. The murine antibody was subsequently detected with a rabbit anti-mouse antibody before incubation with a TRITC-conjugated swine anti-rabbit antibody (Dako). Between each incubation step and before addition of fluorescent mounting medium (Dako) slides were washed four times in PBS supplemented with 0.05% Tween 20 and four times in PBS. Cells were analyzed by confocal laser scanning microscopy using a Leica DM IRBE microscope equipped with TCS system. Images were subsequently prepared using Adobe Photoshop 5.0.

Sequencing—The variable genes from individual clones were amplified by PCR and sequenced as described elsewhere (18). The gene segments were identified using the VBASE directory at the Medical Research Council, Cambridge, UK.3

RESULTS AND DISCUSSION

Selection of Antibodies Directed against Extracellular Epitopes of Cultured Human Keratinocytes—Human keratinocytes were chosen as the target for several reasons. First, they constitute a substantial part of our largest organ, the human skin, and second, only limited information exists on the membrane-associated contents. Third, primary cultures of keratinocytes have not undergone any transformational changes as is the case for the cell lines used in previous studies with phage display in direct selections (13, 14, 30–32). Thus, the cultured cells do to some extend reflect the situation in the skin. Fourth, keratinocytes are grown in a defined medium without the addition of fetal calf serum thereby avoiding selection of antibodies recognizing serum proteins, which is observed when traditional selections are performed on cells grown in medium supplemented with fetal calf serum.4 Furthermore, preliminary studies showed that it was not necessary to fix the cells before selection due to the adherent capacity of this particular cell type (data not shown); the antigen conformations consequently will not be modified by applied fixation procedures. Fixation prior to selection has been shown to result in a higher retrieval of specifically binding phage (33), but the retrieved antibodies were of limited applicability (34).

Phage display using phagemid systems relies on the addition of structural proteins from a replicative unit, a helper phage needed for the production of phage particles, while phage are isolated on the basis of fusion coat protein IIIs encoded by phagemids. The helper phage KM13 (17) contains an engineered coat protein III in which a protease-sensitive linker is inserted between the domain anchoring the protein into the phage coat, domain III, and the two domains responsible for infection, domains I and II (35). Thus, protease treatment renders helper phage-encoded coat protein IIIs non-functional with respect to infection, whereas phagemid-encoded fusion coat protein IIIs remain functional for infection. This is critical when phagemid repertoires are used in selections in general and in cell surface selections in particular because only a small fraction of phage in a phage pool contains a fusion coat protein III. The magnitude of this fraction very much depends on the type of repertoire. In a synthetic repertoire, such as the Griffin repertoire, ~1/1000 phage display an antibody fragment, whereas the display level of single framework repertoires, like the Tomlinson I and J repertoires, is in the order of 1/10 to 1/100 when colo
ing-forming units are determined for unselected repertoires before and after protease treatment as well as by Western blotting experiments with an antibody directed against filamentous phage protein III domain III (data not shown). Thus, between 90 and 99.9% of the phage present during a selection represent noise as these non-displaying phage still contain an antibody gene and therefore need to be analyzed if they are retrieved after a selection. This observation implies that the use of KM13 in selections will lower the background between 20- and 1000-fold when compared with a traditional selection because nonspecific phage are retrieved due to their presence and not based on a displayed antibody fragment. Accordingly the nonspecifically retrieved phage are a mixture of non-displaying and displaying phage representing the initial pool, and the majority are consequently removed by protease treatment. The high fraction of nonspecifically retrieved antibodies has been a considerable obstacle in cell surface selection (33). Therefore, we set out to apply the protease-sensitive helper phage in cell surface selection. Furthermore, the presence of a Myc tag between the displayed antibody fragment and protein III in the fusion coat protein as well as the protease-sensitive linker in helper phage-encoded protein III enables elution of phage bound via antibodies and concomitant functional elimination of helper phage-encoded protein III (17, 36).

To verify the benefits gained by using the novel helper phage KM13 over the traditionally used helper phage M13K07, two cell surface selections were performed in parallel with the Griffin repertoire. A number of antibodies were screened after both the first and second round of selection (Table I). It was observed that background antibodies were selected with M13K07, whereas KM13 directed the selections toward epitopes that were either cell-associated or secreted.

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3 www.mrc-cpe.cam.ac.uk/DNAPLOT.php?menu=901.
4 P. Kristensen, unpublished data.
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by keratinocytes. Screening showed that 6% and over 60% of the recovered phage were positive after one and two rounds of selection, respectively. Such high fractions of target-specific antibodies have previously only been reported after selections with phage vector-based repertoires (37). However, such phage vector-based repertoires often exhibit much decreased diversities compared with phagemid-based repertoires. Moreover, KM13 can be applied directly with many of the existing phagemid-based repertoires.

The number of infectious phage particles was 1000-fold lower when KM13 was applied in selections. This is as expected due to removal of non-displaying phage, and it reflects the display level of the phage repertoire. The low output facilitates screening in a high throughput manner after one round of selection, which preserves the diversity of the repertoire (Table I). Selections performed with the traditional helper phage M13K07 require iterative rounds of selection since screening of between 10^6 and 10^7 clones is impractical.

Next positive clones were tested in ELISA for recognition of blocking agent, medium, and cells, and those only recognizing cells were scored round, whereas the ratio only increases 10-fold in the M13K07 selections. The indicated number of colonies was tested in ELISA in an initial test for specificity to determine whether the clones were positive. Fifty microliters of monoclonal phage were added to each well in 2% MPBS. Such high fractions of target-specific antibodies have previously only been reported after selections with phage vector-based repertoires (37). However, such phage vector-based repertoires often exhibit much decreased diversities compared with phagemid-based repertoires. Moreover, KM13 can be applied directly with many of the existing phagemid-based repertoires.

Fourteen unique cell-positive antibodies as verified by DNA sequencing, eight from the Griffin repertoire, two from the Tomlinson I repertoire, and four from the Tomlinson J repertoire, were chosen randomly for additional screening from the large panel of isolated antibodies. These antibodies functioned neither as phage antibodies nor as soluble antibodies in Western blotting experiments performed on cell extracts as well as conditioned medium according to Ref. 18. This indicates that structured epitopes are recognized by the antibodies or that there are problems associated with the analysis of certain proteins due to, e.g. the presence of membrane-spanning domains. For a number of the antibodies, binding of phage-displayed antibodies to cultured keratinocytes was affected by oxidation with periodate (data not shown), which suggests that the epitopes recognized involve carbohydrate structures (39, 40).

Specificity for Keratinocytes—A panel of different cell types was screened in ELISA to determine the cellular specificity of the antibodies. As seen in Table II, 6 of 14 antibodies bound keratinocytes preferentially, three of which bound exclusively, and only two of the antibodies bound antigens expressed by all cell types. It is noteworthy that seven of the antibodies did not recognize an SV40-transformed keratinocyte cell line, which shows that the surface of this cell line, although of keratinocyte origin, does not resemble that of a primary culture. A more general notion would be that if the antigen is present on SV40 keratinocytes, then it is also expressed on the other cell types (D4, H3, A4, 52, and 21). These results can be interpreted in one of two ways: first that primary keratinocytes have a very distinct expression pattern or second that there are problems associated with the analysis of certain proteins due to, e.g. the presence of membrane-spanning domains. For a number of the antibodies, binding of phage-displayed antibodies to cultured keratinocytes was affected by oxidation with periodate (data not shown), which suggests that the epitopes recognized involve carbohydrate structures (39, 40).

Table I

<table>
<thead>
<tr>
<th>Repertoire</th>
<th>Helper phage</th>
<th>Selection round</th>
<th>Input titer</th>
<th>Output titer</th>
<th>Output/input</th>
<th>Positive clones</th>
<th>Cell-positive</th>
<th>Diversity</th>
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<td>M13K07</td>
<td>1</td>
<td>2 x 10^13</td>
<td>5 x 10^6</td>
<td>2.5 x 10^-2</td>
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<td>3 x 10^5</td>
<td>3.0 x 10^-6</td>
<td>69/192</td>
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<td>3 x 10^3</td>
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<td>12/29</td>
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<td>6/12</td>
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<td>10^4</td>
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<td>4/4</td>
<td>4/4^b</td>
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<td>2 x 10^11</td>
<td>10^3</td>
<td>2 x 10^-6</td>
<td>76/384</td>
<td>23/76</td>
<td>22/23^b</td>
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</table>

^a BstNI finger printing of PCR products was subsequently used to determine the diversity of cell-positive clones. ^b Sequencing was subsequently used to determine the diversity of cell-positive clones.
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Monoclonal phage ELISA was performed on an equal number of cells of different origin using 50 μl of each monoclonal phage antibody/well. HeLa and MCF-7, human epithelial cell lines; HEK-293, human embryonic kidney cell line; MRC5-V2, human embryo lung fibroblasts; SaOS-2 and KHOS, human osteosarcoma cell lines; SV40 keratinocyte, SV40-transformed keratinocyte cell line; C2C12, murine pluripotent mesenchymal precursor cell line. Fibroblasts, osteoblasts, and stroma cells are all from primary cultures obtained from normal human donors. The different antibodies have been rated according to their signal intensities in ELISA measured at A\textsubscript{490 nm} on the respective cells and subsequently normalized to that observed for keratinocytes. –, background; +, above background and below 25% of that of keratinocyte; ++, between 25 and 50%; ++++, between 50 and 75%; *, above 75%; ND, not determined. All measurements were performed in triplicate, and keratinocyte standards were included on all ELISA plates.

<table>
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<th>26</th>
<th>12</th>
<th>42</th>
<th>E8</th>
<th>19</th>
<th>20</th>
<th>D4</th>
<th>A4</th>
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**Table II**

Specificity test of the obtained antibodies

The selection method makes it possible to identify small differences among cell populations. Moreover, the observed variation in recognition pattern indicates that the antibodies identify different antigens, which is further supported by the observation that all antibodies contained different CDR3 regions and that the antibodies exhibit distinct staining patterns when applied in indirect immunofluorescence (data not shown).

**Soluble High Molecular Weight Proteins as Targets in Selections**—Three antibodies, 42, D4, and 52, recognized antigens secreted to the medium by keratinocytes (Fig. 1). This indicates that these antigens could be pure extracellular proteins or membrane-associated proteins. Two of the clones, 42 and 52, were isolated after the second round of selection. D4 was the only antibody clone of those screened after the first round selection that appears to recognize antigens secreted by keratinocytes. This suggests a strong bias toward antibodies recognizing soluble antigens when performing multiple rounds of selection. Such bias explains past problems in cell surface selections using multiple rounds of selection in combination with the traditional helper phage. One explanation for this phenomenon is that the obtainable antigen concentration is much higher on the plastic surface than compared with that present on the cell surface, which is continuously renewing itself. Accordingly, in cell surface selections, the number of cells limits the antigen concentration.

To further characterize the antigens recognized by 42, 52, and D4, culture medium was obtained from keratinocytes and fractionated using ion-exchange chromatography. After gradient elution all fractions were tested for the presence of antigen. The identified fractions were analyzed by SDS-PAGE, which showed that several different proteins eluted under the same conditions (data not shown). Positive fractions were concentrated and analyzed by gel filtration using HPLC. The antigens of 42 and 52 eluted in the void volume, whereas the cognate antigen of D4 eluted immediately after the void volume. These retention times correspond to protein complexes exceeding 500 and 450 kDa in globular mass, respectively (data not shown). Thus, high molecular weight antigens seem to constitute targets when the described selection method is applied.

**Determination of Antigen Identities by Mass Spectrometry**—Preparative ion-exchange chromatography was performed, and D4-positive fractions were pooled and dialyzed against an acetic acid buffer. This led to massive precipitation of protein. After resuspension, the precipitated protein was found to contain the antigen of D4 and was therefore subjected to precipitation using antibody D4 coated on magnetic beads (Fig. 2). Similar enrichments were not possible for the antigens recognized by antibodies 42 and 52. Furthermore, at
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Fig. 2. Immunoprecipitation with D4-pIII antibody fusion protein. The result of the extraction was analyzed using 8% SDS-PAGE and silver staining. Antibody D4 was coated on Dynal beads (Dynal) according to the manufacturer’s instructions. Twenty microliters of D4-coated beads were incubated overnight with protein extract, and the beads were washed with varying stringency. As a negative control, an anti-laminin antibody, L36-pIII antibody fusion protein (29), was incubated with the protein extract. Lane 1, molecular mass marker indicating 66 and 97 kDa; lane 2, the protein extract applied for extractions; lane 3, beads coated with antibody D4-pIII fusion protein; lane 4, protein extracts after incubation with D4-pIII coated beads; lanes 5–7, protein immunoprecipitated by antibody D4 after increased washing stringency either three times in PBS (lane 5), five times in PBS (lane 6), or two times in PBS supplemented with 1% Triton X-100 followed by three times in PBS (lane 7); lane 8, protein extracts after incubation with L36-pIII-coated beads; lane 9, protein immunoprecipitated by antibody L36 after washing two times in PBS supplemented with 1% Triton X-100 followed by three times in PBS. The band observed at 60 kDa corresponds to the antibody pIII fusion protein. The extra band in lane 9 results from the purification and is not extracted from the protein extract. The high molecular weight protein band was excised, digested in-gel with trypsin, and analyzed by mass spectrometry (41).

The very high molecular weight protein band extracted by B3 was identified as plectin (Table III), a 450-kDa widespread cytoskeletal linker protein found in hemidesmosomes (43). Colocalization studies with a commercial plectin antibody and B3 further substantiated the extraction data (Fig. 4b). Although plectin is a ubiquitously expressed protein, multiple isoforms exist, some with expression pattern in accordance with our results (Table II) (44, 45). Plectin has normally been localized to the cytoplasmic side of the cell membrane, and it would therefore be difficult to isolate phage antibodies by direct selection against such targets. Two different scenarios could explain this observation: (a) given that the localization of plectin is entirely cytoplasmic it is made available for selection when cells are stained without permeabilizing the plasma membrane by the addition of Tween 20 (data not shown). Thus, the presented method is capable of revealing subtle topological structure, an observation that could elucidate the role of plectin as an antigen in autoimmune diseases (46–48). Moreover, other cytoplasmic proteins, e.g. actin and a novel human cytoplasmic protein, p23, in similar phage display experiments have revealed themselves as cell surface-exposed (49, 50).

Antibody 12 immunoprecipitates three proteins as revealed
by SDS-PAGE using reducing conditions. Mass spectrometry analysis and database searching identified these three bands as α3, β3, and γ2 laminin subunits (Table III). The apparent molecular weights of the precipitated proteins observed by SDS-PAGE correspond to the calculated molecular weights determined from the amino acid sequences of these laminin subunits. However, α3 is extracted in an unprocessed form. These three protein subunits are the constituents of laminin-5, a laminin isoform found in the basement membrane of the dermal-epidermal junction (51, 52). Epithelial cells secrete laminin-5; notwithstanding, we did not detect significant amounts in the culture medium (Fig. 1). It has previously been shown that laminin-5 binds with high affinity to its receptors, integrin α3β1 and α6β4, and that only a minor fraction can be found free in the medium, supporting this observation (53). ELISA with purified recombinant laminin-5 verified the immunoprecipitation and mass spectrometry data (Fig. 4). Antibodies recognizing matrix molecules in their natural environment could be applied in therapy as these potentially have an anti-invasive tumor activity (54).

Conclusion—In recent years, phage display has established itself as an alternative method for the generation of monoclonal antibodies against individual antigens as well as components of complex mixtures. Here we have described the development and improvement of selection strategies to diversify the targeted structures in quests for cell markers using three different phage-displayed antibody repertoires. Implementation of novel methodological improvements facilitated isolation of antibodies recognizing cell markers using only a single round of selection in contrast to the traditional use of iterative rounds of selection and testing of a few highly enriched clones. Screening after the first round of selection preserves the diversity of antibodies, thereby targeting the broadest possible range of antigens. This is of significant importance because biologically relevant antibodies may not bind with high affinity and may therefore be lost during multiple rounds of selection. Furthermore, the combination of immunoprecipitation with mass spectrometry allowed the identification of three proteinaceous antigens, laminin-5, plectin, and fibronectin, that were subsequently validated using

### TABLE III

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein</th>
<th>Swiss-Prot accession no.</th>
<th>Number of peptides matched</th>
<th>Sequence coverage %</th>
<th>Expectation value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4</td>
<td>Fibronectin Precursor</td>
<td>P02751</td>
<td>28</td>
<td>21</td>
<td>2.5 × 10⁻⁷</td>
</tr>
<tr>
<td>B3</td>
<td>Plectin</td>
<td>Q15149</td>
<td>61</td>
<td>16</td>
<td>7.9 × 10⁻⁶</td>
</tr>
<tr>
<td>12.A</td>
<td>Laminin-5 α3B chain</td>
<td>X84900</td>
<td>25</td>
<td>20</td>
<td>2.6 × 10⁻⁵</td>
</tr>
<tr>
<td>12.B</td>
<td>Laminin γ2 chain</td>
<td>gi</td>
<td>5777581</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>12.C</td>
<td>Laminin β3 chain (laminin-5 β3)</td>
<td>Q13751</td>
<td>18</td>
<td>21</td>
<td>1.6 × 10⁻⁶</td>
</tr>
</tbody>
</table>

- **a** Number of matching tryptic peptide masses with a mass error of less than 50 ppm.
- **b** The percentage of amino acid residues recovered by mass spectrometry out of the total number of amino acids in the protein.
- **c** ProFound expectation value.

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FIG. 4. Verification of antigen identities. *a*, ELISA was performed on purified recombinant laminin-5 (black bars), purified cellular fibronectin (gray bars), and milk proteins (white bars) with phage-displayed antibodies. The absorbance was subsequently read at 490 nm. *b*, co-localization studies were performed on cultured epidermal keratinocytes with biotinylated antibody B3 (green color) as well as a commercially monoclonal plectin antibody (red color). Co-localization is observed as the yellow color. Complete co-localization is observed for these two antibodies throughout the cell as seen in the six serial optical sections (numbered consecutively from 1 to 6) indicating that the antibodies recognize antigens in the same cellular compartments. Stained cells were studied by confocal laser scanning microscopy.
complementary methods. Our observation that plectin is exposed as a target during the selection implicates that a fraction of plectin molecules are localized at the cell surface. This indicates that the cellular microenvironment is potentially much more complex since de facto cytoplasmic proteins can be found on the cell surface.

Phage display is emerging as a promising and useful technique in the expanding field of proteomics (21, 38, 55), although it is still in its infancy in this respect. The postgenomic era of molecular and cellular biology calls for methods that are capable of analyzing and identifying differentially expressed proteins to analyze the complexity of the functional genome. The most prominent feature of phage display is the ability to isolate ligands, in particular antibodies, with affinity for the target and subsequently analyze these with respect to their specificities. Thus, phage display is a supplement to already existing tools, and it has been applied in the quest for novel cell markers. The technique of phage display will certainly prosper by implementation of further improvements. For example, it still needs to be determined whether phage display selections obey the mass action law of thermodynamics or whether antibodies are selected by chance. As the first case is the most likely, the sensitivity of phage-displayed selections could be insufficient to analyze the vast majority of proteins expressed by the cells as identification of low abundance antigens requires the availability of high affinity antibody repertoires. The applicability of phage display–based selection methods in proteomic research will gain from further improvements to increase the sensitivity as described in the present study as well as availability of additional high quality repertoires. The quality of repertoires can be improved by enhancing the functionality because a general increase in the amount of each antibody will improve the chances of isolating high affinity as well as low affinity antibodies according to the mass action law. Nonetheless, our results indicate that the semisynthetic Griffin repertoire performs just as well in selections as the single framework Tomlinson repertoires with an increased functionality as determined by the display level. Thus, the display level does not necessarily correlate with the performance of repertoires in selections. Therefore it can be speculated whether antibodies are selected by chance. Alternatively, semisynthetic repertoires based on panels of different antibody genes could target a broader range of antigens in complex selections due to greater variation in antibody structures.

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