Profiling of Alopecia Areata Autoantigens Based on Protein Microarray Technology*

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Protein biochips have a great potential in future parallel processing of complex samples as a research tool and in diagnostics. For the generation of protein biochips, highly automated technologies have been developed for cDNA expression library production, high throughput protein expression, large scale analysis of proteins, and protein microarray generation. Using this technology, we present here a strategy to identify potential autoantigens involved in the pathogenesis of alopecia areata, an often chronic disease leading to the rapid loss of scalp hair. Only little is known about the putative autoantigen(s) involved in this process. By combining protein microarray technology with the use of large cDNA expression libraries, we profiled the autoantibody repertoire of sera from alopecia areata patients against a human protein array consisting of 37,200 redundant, recombinant human proteins. The data sets obtained from incubations with patient sera were compared with control sera from clinically healthy persons and to background incubations with anti-human IgG antibodies. From these results, a smaller protein subset was generated and subjected to qualitative and quantitative validation on highly sensitive protein microarrays to identify novel alopecia areata-associated autoantigens. Eight autoantigens were identified by protein chip technology and were successfully confirmed by Western blot analysis. These autoantigens were arrayed on protein microarrays to generate a disease-associated protein chip. To confirm the specificity of the results obtained, sera from patients with psoriasis or hand and foot eczema as well as skin allergy were additionally examined on the disease-associated protein chip. By using alopecia areata as a model for an autoimmune disease, our investigations show that the protein microarray technology has potential for the identification and evaluation of autoantigens as well as in diagnosis such as to differentiate alopecia areata from other skin diseases. ***Molecular & Cellular Proteomics 4:1382–1390, 2005.***

Autoimmune diseases affect 5% of the world population, and our understanding and treatment of human autoimmune diseases has to be improved (1). Often the current diagnostic tools are limited because there is no assay to detect the quality of the patients' response to drugs. Thus, in addition to improved diagnostics the discrimination between drug responder and drug non-responder before the onset of therapy might be a goal for future strategies in the treatment of autoimmune diseases.

A characteristic feature of many autoimmune diseases is the production of autoantibodies (2). Although the pathogenic role for most of the autoantibodies in various autoimmune diseases is not clear, the identification of the autoantigens that are targeted by the autoantibodies during the immune response may present an important tool for diagnosis, classification, and prognosis. Additionally profiling the autoantibody repertoire may help to elucidate the pathophysiology of autoimmunity, enabling novel treatments such as antigen-tolerating therapy (3, 4). Protein microarrays have been used for detection and validation of autoantibodies in biological fluids (5, 6).

Alopecia areata is an autoimmune disease of the hair follicles that affects between 2 and 4% of patients seen in dermatological practice (7) resulting in rapid loss of hair, often featured by nail dystrophy. The loss may reverse completely, become chronic, or progress to loss of all scalp hair (alopecia totalis) and all body hair (alopecia universalis). It is diagnosed by the clinical finding of a patchy hairless area with some dystrophic hairs called "exclamation point" hairs at the border of the patch. It may be confirmed by characteristic histological findings of a dense peri- and intrabulbar inflammatory infiltrate of anagen hair follicles like a bee swarm. The disease is characterized by a frequently relapsing course with good response to systemic immunosuppression (corticosteroids) or topical immunomodulation with diphencyprone (8).

Progress has been achieved over the past 5–10 years in the characterization of hair follicle antigens targeted by antibodies in alopecia areata (9). Candidate autoantigens that have been identified include the 44/46-kDa hair-specific keratin and trichohyalin. Moreover there is evidence that anti-hair follicle antibodies are modulated during the disease process and can

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occur before clinically evident hair loss (9). In addition, differentiating anagen keratinocytes are an important structure in the autoimmune etiology of alopecia both in autoimmune endocrine syndrome type I-associated alopecia areata and at least in a subgroup of patients with alopecia areata unrelated to autoimmune polyendocrine syndrome type I (10).

There is evidence for a polygenic inheritance, and diverse candidate genes have so far been identified by case-control and family-based studies: HLA class II genes (11), interleukin-1 cluster genes (12, 13), and polymorphism in the interferon-induced p78 protein MxA, which is highly expressed in anagen hair bulbs of alopecia areata lesions (14).

In this study, we combined protein microarray technology with the concept of an arrayed expression library enabling direct connection from the protein product of an individual expression clone to its corresponding cDNA sequence information (for reviews, see Refs. 15–17). Using robot technology, a human fetal brain cDNA expression library (hEx1) was picked into microtiter plates, and high density protein arrays were produced on filter membranes followed by *in situ* expression (18). In an initial analysis of this library, more than 66% of the clones contain inserts in the correct reading frame. 64% of these clones comprise full-length proteins (5, 19). Based on an oligonucleotide fingerprinting analysis, ~13,000 different genes have been determined in this expression library indicating a high variance of presented proteins. Therefore, this library seems to be highly suitable for unbiased screening assays.

Screening protein arrays with body fluids from large numbers of patients with an autoimmune disease would allow the identification of potentially novel autoantigens as well as potentially assist the prognosis, diagnosis, and subtyping of autoimmune diseases based on the presence of specific autoantibodies. In diagnostic applications denatured proteins are preferentially used due to their higher stability during storage. Because our study has the aim to develop a disease-associated protein chip suitable for diagnosis, we have used denatured proteins and focused on autoantibodies recognizing linear epitopes. Peptides, which present linear epitopes, have been successfully used to profile the autoantibody repertoire (6, 20, 21).

Here we used alopecia areata as a model for a T-cell-mediated autoimmune disease and profiled the autoantibody repertoire of alopecia areata patients. Following identification of putative autoantigens, we extended the recently described approach by arraying the identified putative autoantigens on protein microarrays to generate a disease-associated protein chip to specify and differentiate between these candidate autoantigens. To further confirm the specificity of the results obtained, sera from patients with psoriasis or hand and foot eczema were additionally analyzed on the disease-associated protein chip.

EXPERIMENTAL PROCEDURES

Patient Data—24 patients with alopecia areata (12 alopecia areata circumscripta, two alopecia areata of diffuse type, seven alopecia areata subtotalis or universalis, one alopecia areata ophiasis type, two alopecia areata data not available) in an active stage without any systemic or topical therapy were randomly chosen for participation in this investigation. The patient control group consisted of patients with normal hair growth and no personal or family history of alopecia areata. The disease control group consists of sera that were collected from patients with hand and foot or atopic eczema ($n = 5$) and psoriasis ($n = 4$) at a mean age of 38 years.

The alopecia areata patients, of which 7 of 24 were male (29%) and 17 of 24 were female (71%) at a mean age of 35 years in male and 38 years in female, agreed to donate a blood sample after having received written information and signed the informed consent. 2 of 7 (30%) of the male population had atopic diathesis, whereas 8 of 18 (47%) of the female population had atopy. Autoimmune parameters (antinuclear antibodies (ANA)[1] and autoantibodies against TSH receptor (TRAK)) were analyzed in addition. The patient population showed in total in 9 of 24 (38%) a positive antinuclear antibody level (22% in male and 16% in female), and in 6 of 24 patients (25%) antibodies against thyroid were found to be positive. All patients showed IgG levels in the normal range. One patient also was diagnosed to suffer from vitiligo. This study was accredited by the Charité ethics commission. Blood was collected from alopecia areata patients in BD Biosciences Vacutainer systems. After centrifugation at 3000 rpm/min serum was distributed and analyzed in the central clinical chemistry unit at the Institute of Clinical Chemistry and Pathobiochemistry, Charité Campus Benjamin Franklin for the following parameters: IgG, IgE, ANA, autoantibodies against thyroid peroxidase (anti-TPO), and TRAK. IgG was quantified on a Behring NephelometerII® by a mechanized nephelometric assay (Dade Behring). IgE was determined on a UniCap 100® analyzer by a mechanized fluorescence enzyme immunoassay (Sweden Diagnostics). ANA was determined manually by indirect autoimmunofluorescence microscopy. Anti-TPO was analyzed on a Kryptor® analyzer with an automated immunofluorescence assay from BRAHMS Diagnostica using time-resolved amplified cryptate emission technology, TRAK was quantified with a luminescence receptor assay with a coated tube system based on the anti-TSH receptor autoantibody-mediated inhibition of binding of labeled TSH to the TSH receptor.

Expression Vector and Bacterial Strain—A protein expression subset of a cDNA library (hEx1) from human fetal brain cloned in the protein expression vector pQE30NST (GenBank™ accession number AF074376) and transformed into the *Escherichia coli* strain SCS1 (Stratagene) (18), which consisted of 37,200 clones, was used for autoantibody profiling.

Sequence Analysis of Expression Clones—cDNA inserts were PCR-amplified and tag-sequenced as described previously (19). The sequences were searched against public databases (National Center for Biotechnology Information (NCBI)) (22).

Serum Profiling on High Density Protein Arrays—High density protein arrays of the protein expression set of the hEx1 library were obtained from the German Resource Center for Genome Research (RZPD) and prepared as described previously (18). For serum profiling, the filters were blocked in 3% (w/v) nonfat, dry milk powder in TBST (TBS, 0.1% (v/v) Tween 20) for 2 h, washed twice in TBST, and subsequently incubated with serum pools, each containing four patient sera adjusted to identical IgG levels (10 μg/ml) and diluted 1:20 in 2% (w/v) BSA, TBST for 16 h. Following three 30-min TBST washes

[1] The abbreviations used are: ANA, antinuclear antibodies; TRAK, autoantibodies against TSH receptor; TSH, thyroid-stimulating hormone; anti-TPO, autoantibodies against thyroid peroxidase; AP, alkaline phosphatase; CV, coefficient of variation; FGFR3, fibroblast growth factor receptor 3; DCM, dilated cardiomyopathy; EPF, endemic pemphigus foliaceus; SLE, systemic lupus erythematosus; E2, ubiquitin carrier protein; DC, disease control.

and subsequent incubation with the secondary antibody (either mouse anti-human IgG, Sigma, 1:5000 dilution or mouse anti-human IgG3, Sigma, 1:400 dilution as appropriate) in 2% (w/v) BSA, TBST, the filters were washed three times for 30 min in TBST. This was followed by incubation with the tertiary antibody (rabbit anti-mouse IgG alkaline phosphatase (AP)-conjugated, Sigma, 1:5000 dilution) in 2% (w/v) BSA, TBST. Subsequently the filters were washed three times in TBST-T (TBST, 1% Triton X-100) for 20 min each followed by a 10-min wash in TBS and a further wash for 10 min in AP buffer (1 mM $MgCl_2$, 0.1 M Tris, pH 9.5) and subsequent incubation in 25 mM Attophos (JBL Scientific) in AP buffer for 5 min. The filters were illuminated with long wave UV light, and the images were taken using a high resolution charge-coupled device detection system (Fuji). Image analysis was performed with VisualGrid (GPC Biotech, Munich, Germany).

Protein Expression and Purification in High Throughput—Each protein was expressed in 1-ml cultures in deep well microtiter plates. The proteins were extracted from each culture (23) and purified as described previously (19).

Generation of Protein Microarrays—FAST slides (Schleicher & Schuell) were placed in a Q-Array System (Genetix, New Milton, UK) equipped with humidity control (65%), and 16 or 24 blunt-ended stainless steel print tips with a tip diameter of 150 μm were used to generate the protein arrays. All protein antigens were spotted in duplicate onto two fields in three different concentrations (undiluted and 1:5 and 1:10 diluted). For most of the proteins, spotting of the undiluted purified proteins leads to immobilization of 15 fmol of protein/spot on the microarray (undiluted: 15 fmol; 1:5: 3 fmol; 1:10: 1.5 fmol, respectively). Each protein microarray included several control spots, such as human or mouse serum IgG in three concentrations (human IgG: 1:1000, 1:2500, and 1:5000; mouse anti-human IgG: 1:1000, 1:2500, and 1:5000).

Serum Profiling on Protein Microarrays—After spotting, the protein chips were blocked in 2% (w/v) BSA, TBST, 0.1% (v/v) Tween 20 at room temperature, and the serum was added in a 1:100 dilution in 2% (w/v) BSA, TBST. The protein chips were incubated in a humidified atmosphere for 16 h at 4 °C. Following three 30-min TBST washes and subsequent incubation with the secondary antibody (mouse anti-human IgG, Sigma, 1:5000 dilution) in 2% (w/v) BSA, TBST, the protein chips were washed three times for 20 min in TBST. This was followed by incubation with the tertiary antibody (rabbit anti-mouse IgG-Cy3) in 2% (w/v) BSA, TBST. Subsequently the chips were washed three times each in TBST for 20 min. All secondary and tertiary antibody incubation steps were performed for 1 h at room temperature and carried out in a volume of 200 μl underneath a cover slide in the dark. These protein chips were imaged using a confocal microarray reader (ScanArray 4000, PerkinElmer Life Science), and image analysis was performed using ScanArray Express (PerkinElmer Life Sciences). One protein microarray from each batch of the spotted chips was incubated with an antibody directed against the amino-terminal RGS His_6 tag of the immobilized recombinant protein. The resulting intensities, which reflect the corresponding protein concentration, were used for normalization by dividing each intensity value from the serum incubation by the corresponding RGS His_6 intensity value.

Western Immunoblot Analyses—For Western blot analyses, proteins were purified under denaturing conditions from 300 ml of bacterial cultures grown at 37 °C. Expression of proteins fused with a His_6 tag was induced in the cultures with 1 mM isopropyl β-D-thiogalactopyranoside at an A_{578} of 0.6–0.7. After 4 h cells were pelleted, resuspended in 10 ml of buffer B (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris/HCl, pH 8.0) and incubated at 4 °C for 1 h. Poly-Prep® chromatography columns (Bio-Rad) containing 0.5 ml nickel-nitrilotriacetic acid-agarose (Qiagen) were equilibrated twice with 10 ml of buffer B. After centrifugation, 10 ml of the cell lysate were loaded onto the columns, which were subsequently washed three times with 10 ml of buffer C (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris/HCl, pH 6.3). Elution was performed with 5 ml of buffer E (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris/HCl, pH 4.5).

100 ng of each protein were separated by SDS-PAGE and transferred to PVDF membrane (PolyScreen®, PerkinElmer Life Sciences) by semidry blotting. The membrane was blocked in TST buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 h and incubated for 1 h with serum adjusted to 10 μg/ml IgG in TST (alopecia areata patients serum and control patients serum) or diluted 1:1000 in TST (control human serum). After three washings in TST, the membranes were incubated for 30 min with goat anti-human IgG-horseradish peroxidase antibody (Dianova) diluted 1:10,000 in TST and subsequently washed in TST. Chemoluminescence detection was performed with a FujiFilm LAS-1000 imager by scanning membranes treated with Lumi-Light® Western blotting substrate (Roche Applied Science).

Bioinformatic Analysis—Following the image analysis the mean intensity (median background subtracted) was determined for each protein feature.

To determine putative biomarkers, the average value of the protein duplicates or quadruplicates was calculated ($average_{protein}$ of patient $serum_n$ or control $serum_n$ value) followed by the determination of the quality of the screen by calculating the coefficient of variation of the control antibodies, human IgG and mouse IgG, respectively. These values were also used for interchip normalization. To determine and consider differences of protein chip production, each chip from a screen was normalized against the RGS His_6 epitope tag intensity, which reflects the corresponding protein concentration. The mean value of all average intensities of the control serum group ($average_{protein}$ of control $serum_n$ values) was calculated that is called the "$average_{protein\ X}$ control" value. Dividing the average value of each individual patient serum (named $average_{protein\ X}$ patient $serum_n$ value) by the "$average_{protein\ X}$ control value" results in the so-called factor F. We suggested F values above 2 for disease-significant autoantigens.

RESULTS AND DISCUSSION

Profiling the Antibody Repertoire of Alopecia Areata Patients—In this study we screened a human high density protein array containing 37,200 redundant, recombinant proteins derived from a human fetal brain expression library with sera obtained from 20 alopecia areata patients. We generated five serum pools, each containing four patient sera, and incubated these serum pools with the high density protein filters. The use of five serum pools instead of higher numbers of individual samples reduced the requirements of materials, *e.g.* the high density protein arrays, as well as of time and labor.

The data sets obtained from screenings of the five serum pools were compared with data sets obtained from screenings of 11 individual control sera from clinically healthy persons and to background incubations with anti-human IgG. From these data, proteins reacting with antibodies present in alopecia areata patient serum pools were identified. When we considered proteins that were detected by antibodies from two or more of the five patient pools but not by antibodies from the control sera, we identified a subset of 23 proteins. 5′-tag sequencing of these clones was performed, and their sequences were used for BLAST searches against the public databases including GenBank™ and Unigene (22).

TABLE I

Analysis of putative alopecia areata specific autoantigens

The column "Hits in pools" shows the numbers of pools detecting the corresponding autoantigens, whereas the column "Hits in Microarrays" shows the numbers of single sera detecting the corresponding autoantigen. The column "Factor Ø" describes the average detection levels obtained from the microarray experiments with patient sera compared to microarray experiments obtained from control sera. The column "ORF" gives information about a correct open reading frame of the antigen detected. EGF, epidermal growth factor.

Clone ID MPMGp800 . . .	Accession no. (GenBank™)	Gene name	Hits in pools (percentage)	Hits in microarrays (percentage)	Factor Ø	ORF
K18585	AV652428	cDNA clone GLCDAC05	4 (80%)	12 (50%)	2	+
M10510	XM031401	EGF-like domain, multiple 3 (EGFL3)	3 (60%)	11 (46%)	2	−
J01523	AK022755	cDNA FLJ12693 fis, clone NT2RP1000324	2 (40%)	19 (79%)	5	+
O22528	NM004436	Endosulfine α (ENSA)	2 (40%)	15 (63%)	5	+
K24594	NM003134	Signal recognition particle subunit 14	2 (40%)	12 (50%)	3	+
D04547	NM022965	FGFR3	2 (40%)	18 (75%)	6	+
O05529	M91670	Keratinocyte ubiquitin carrier protein (E2-EPF)	2 (40%)	17 (71%)	5	+
M17541	BC006318	Erythrocyte membrane protein band 4.9 (dematin)	2 (40%)	20 (83%)	5	+
B20572	NM007029	Neuron-specific growth-associated protein (SCG10)	2 (40%)	12 (50%)	11	+
F11552	NM006769	LMO4	2 (40%)	18 (75%)	17	−

Validation of Putative Autoantigens on Protein Microarrays—The 23 recombinant proteins selected above were expressed from the bacterial clones and purified, in parallel, under denaturing conditions. This corresponded to the state of the proteins immobilized on the PVDF protein array. In addition, 10 randomly chosen proteins that were only detected once from the pooled serum screenings were included. This strategy allowed evaluation of the relevance of the data obtained by screening the patient serum pools. In addition, two proteins representing stathmin or a fragment thereof, which is an antigen of a naturally occurring autoantibody (24), were included to ascertain the quality of the sera used. Following high throughput expression and purification, recombinant proteins were used in three concentrations for the generation of protein microarrays. To enable inter- and intrachip image analysis as well as process and batch control, human IgG (in the form of human serum) and mouse IgG (in the form of mouse anti-human IgG) were included in the chip design.

The protein microarrays were incubated separately with sera from 24 alopecia areata patients including the 20 patient sera examined in the initial high density protein filter screening. In addition, the 11 control sera used in the initial filter screening were also incubated separately on the protein microarrays. To determine the quality of the serum screening, the coefficient of variation (CV) of the process control antigens, namely human IgG and mouse IgG, was determined for each incubated microarray, and the average CV was then calculated. The analysis of both human IgG and mouse IgG had a low CV (1:1000, 1:2500, and 1:5000 dilutions: 9, 22, and 19% for human IgG and 15, 25, and 33% for the mouse IgG, respectively) indicating a comparable quality between patient and control serum incubations.

By bioinformatic analysis, significant differences between patient and control sera were determined for 10 proteins (Table I). Only two of these proteins were not in the same reading frame as the RGS His_6 epitope tag. Sequence searches and comparisons using the amino acid sequences obtained for the reading frames of these two proteins did not allow the identification of a putative autoantigen. This indicates that our presented strategy has a strong bias to identify real existing proteins as potential biomarkers. As seen in Table I, these proteins were all detected by two or more serum pools, whereas proteins only detected by one serum pool were not regarded as significant. However, we found several differences in the percentages between pools and microarray results. These differences may result from the lower numbers of pools, which result in less precise percentages when compared with the higher number of percentages in the microarray experiments. In addition, the experiments based on microarrays are more sensitive and precise because they work for example with purified and concentrated proteins.

To further confirm the presence of autoantibodies against the putative autoantigens identified in the pool screenings, these proteins were expressed and purified in larger scale and analyzed on Western blots with patient sera. Autoantibodies against each of the antigens (except the FGFR3 protein) identified in the screen were detectable in the Western blot analyses as shown for some examples (Fig. 1*B*). Serum from patient P1 gave high overall background and did not allow detection of autoantigen bands. Comparison of the results obtained with high density arrays and Western blots, however, did not show complete correlation of detection of the different putative autoantigens (also compare Fig. 3, *A* and *C*, for alopecia areata patient and Fig. 3, *B* and *D*, for disease control). This could be explained by the presence of serum autoantibodies reacting with epitopes on the microarrays that may have partially refolded, but these antibodies no longer detect the antigen following SDS-PAGE and Western blotting as can be seen for the FGFR3 protein. Otherwise epitopes have to be completely unfolded (*e.g.* by SDS) to be detected.

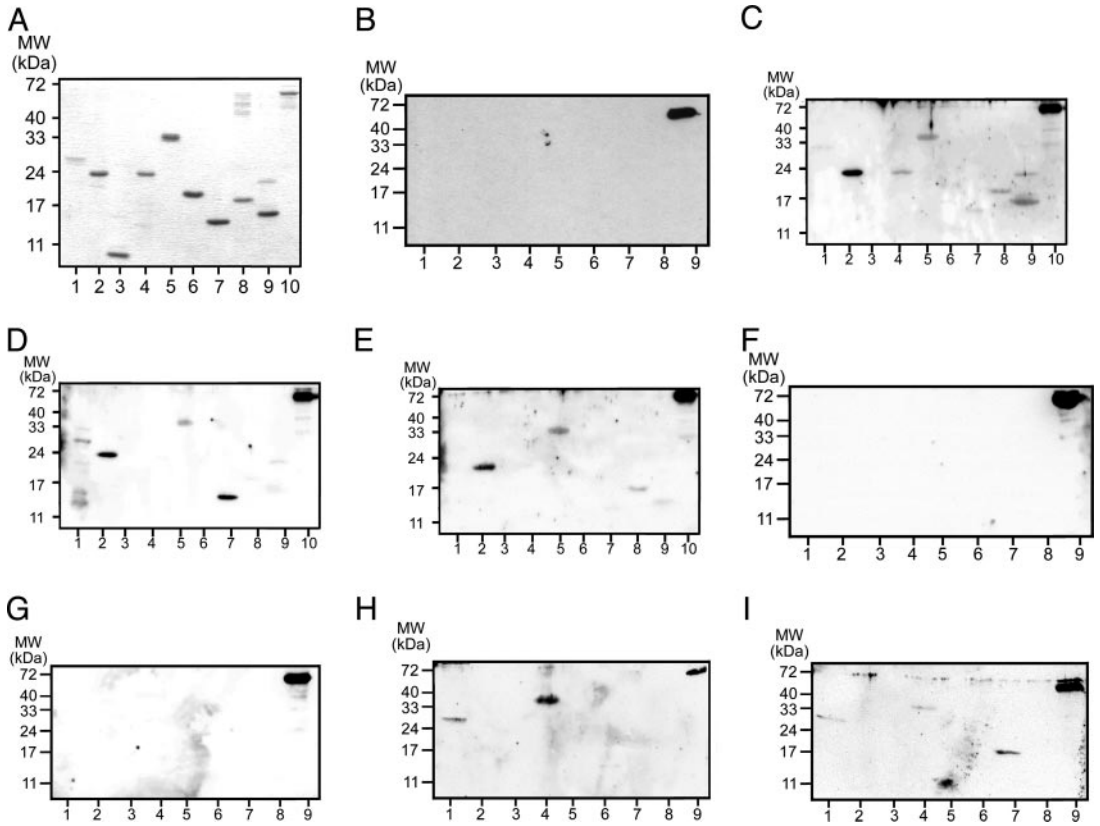

FIG. 1. **Representative Western blot analysis of the putative autoantigens with sera from alopecia areata patients.** *A*, Coomassie-stained input gel showing the putative autoantigen proteins used for Western blot analysis. *B*, control blot using the secondary anti-human IgG peroxidase antibody. *C–E*, Western blot analysis with patient sera P12, P16, and P17. *F* and *G*, Western blots incubated with sera from control persons without obvious skin disease. *H* and *I*, analysis of sera from patients with psoriasis (*H*) and neurodermatitis (*I*) (also see Fig. 3*D*, *DC2* and *DC5*). *A–E*, *lane 1*, SCG 10; *lane 2*, EGI-like domain, multiple 3 in wrong reading frame; *lane 3*, GLCDAC05; *lane 4*, α-endosulfine; *lane 5*, NOL8; *lane 6*, FGFR3; *lane 7*, dematin; *lane 8*, signal recognition particle subunit 14; *lane 9*, EPF autoantigen; *lane 10* human IgG loading reference. *F–I*, loading as in *A–E* with EGI-like domain, multiple 3 omitted.

In a previous study, the IgG- and IgG3-specific antibody repertoire of dilated cardiomyopathy (DCM) patients was profiled (25). In this study, 10 single sera were screened against the human protein filter array containing 37,200 human proteins, and 48 IgG-specific and 32 IgG3-specific autoantigens were identified. The quantitative validation using highly sensitive protein microarrays leads to the confirmation of 10 IgG- and 6 IgG3-specific autoantigens. In the alopecia areata study, we used five serum pools, each containing four different sera from alopecia areata patients, and we identified only 23 putative autoantigens on the human protein filter array. When compared with the DCM study, the number of identified putative autoantigens is remarkably lower. However, at least eight of these proteins with in-frame amino acid sequences were confirmed using both highly sensitive protein microarrays and Western blots leading to similar results when compared with our previous DCM study. This indicates that the use of pools of sera, instead of single serum incubations, is a reliable method for the first screening step on the human filter arrays. We cannot rule out that by using serum pools additional putative autoantigens will/may remain unidentified. However, we expect that the most predominately existing putative autoantigens will be detected by this method. For the development of a disease-associated protein chip, we chose just those proteins that were correctly expressed (correct reading frame with respect to the RGS His_6 epitope) and confirmed by Western blotting.

Development of a Disease-associated Protein Microarray Suitable for Diagnosis of Alopecia Areata Patients—Eight autoantigens were identified by protein chip technology and successfully confirmed by Western blot analysis. These autoantigens were arrayed on protein microarrays to generate a disease-associated protein chip that may be suitable for fast diagnosis. Two control proteins representing natural autoantigens (24) were included as well as human and mouse IgG allowing process control and determination of serum quality (Fig. 2*A*).

The protein microarrays were incubated with a selection of 20 of the currently present 24 patient sera. These incubations were compared with incubations with 10 new collected control sera. To further confirm the specificity of the results obtained, sera from patients with psoriasis or hand and foot eczema as well as skin allergy (so-called disease control sera)

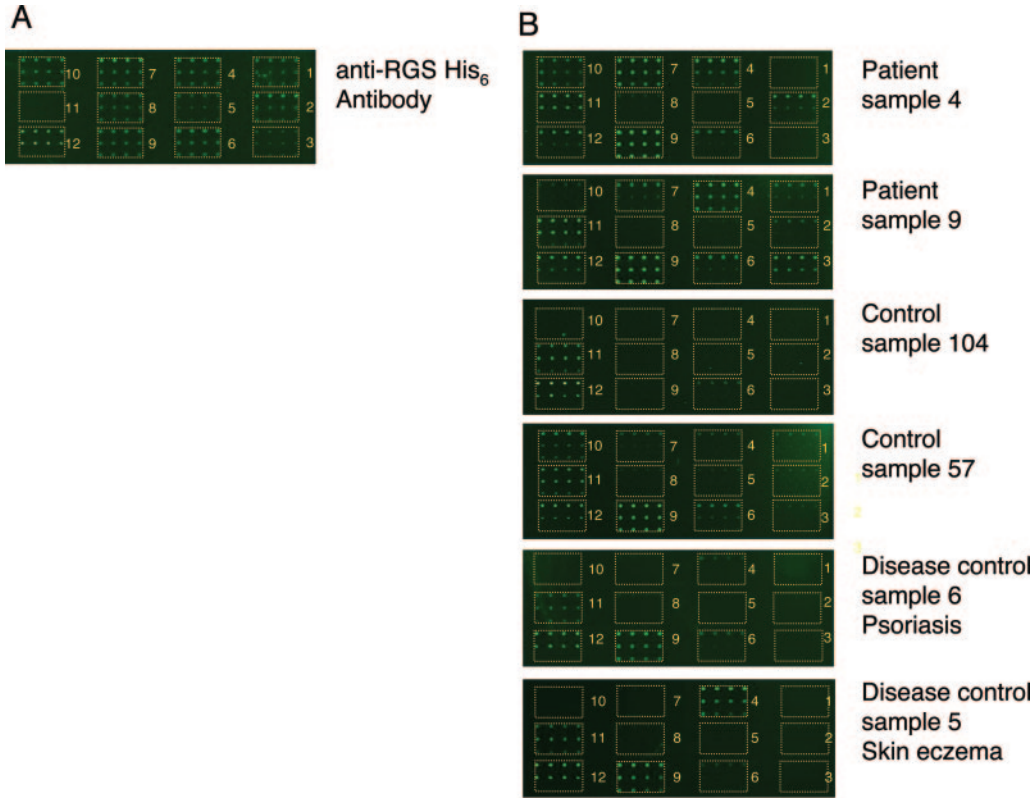

FIG. 2. **Development of a disease-associated protein chip suitable for diagnosis of alopecia areata patients.** *Fields 1–8*, putative autoantigens (*1*, cDNA clone GLCDAC05; *2*, cDNA FLJ12693 fis, clone NT2RP1000324NOL8; *3*, α-endosulfine; *4*, signal recognition particle subunit 14; *5*, FGFR3; *6*, endemic pemphigus foliaceus autoantigen; *7*, dematin; *8*, SCG10); *fields 9* and *10*, natural occurring autoantigens (control proteins; stathmin); *field 11*, human IgG; *field 12*, mouse IgG (process control proteins). All proteins in *fields 1–12* were spotted in quadruplicates in three different concentrations. In addition, 96 randomly selected proteins are spotted that serve as targets for unspecific cross-reaction in case of highly concentrated autoantibodies in the serum. *A*, protein chip incubated with an anti-RGS His_6 antibody to ensure quality of the chip. *B*, diagnostic fields incubated with different samples.

were additionally analyzed on the disease-associated protein chip (Fig. 2*B*). The whole experiment was performed in two independent batches of arrays.

The analysis of the human IgG as well as the mouse IgG showed a low CV (batch 1: 32, 19, and 26%, batch 2: 8, 9, and 24% for human IgG; batch 1: 27, 31, and 17%, batch 2: 8, 7, and 34% for the mouse IgG, respectively) indicating good reproducibility between patient, control, and disease control serum incubations.

When compared with the group of control sera (using average intensity values of each protein and protein concentration), 18 patient sera could be characterized by the presence of autoantibodies against two or more of the chosen biomarker proteins (average, 3.4 recognized autoantigens). One patient showed the presence of autoantibody to one of the selected proteins, and in another patient, serum autoantibodies against the chosen biomarkers were completely missing. In Western blot analyses, however, autoantigens in the serum of this patient could be detected suggesting that this serum was perhaps altered either during transportation or storage. Consequently using the disease-associated protein chip, we could successfully determine 90% (relevance criteria, >2 autoantigens detected) of the alopecia areata patients having this disease (Fig. 3*A*). Comparison of sera from patients with other skin diseases (psoriasis, neurodermatitis, and hand-foot eczema) designated as disease control (DC) group with the control group showed that the disease control sera recognized 1.8 autoantigens on average. In detail, no disease control serum recognized more than three potential autoantigens. Three (of seven) disease control sera recognized three autoantigens, two disease control sera reacted against two proteins, and two disease control sera had no autoantibodies against the chosen biomarker panel (Fig. 3*B*). This indicates that there are antibodies in disease control sera directed against the putative alopecia areata-specific autoantigens. Especially the NOL8 antigen appears to be a more general autoantigen in skin diseases. Similar results were obtained by Robinson *et al.* (6) who have shown that several autoantigens such as Ro52 or histone H2A were recognized by more than one autoimmune disease (Sjögren syndrome, SLE, and mixed connective tissue disease) (6).To address this issue, we suggest the analysis of more patient samples of alopecia areata and other dermatological diseases as a control, which may lead to the identification of certain protein panels character-

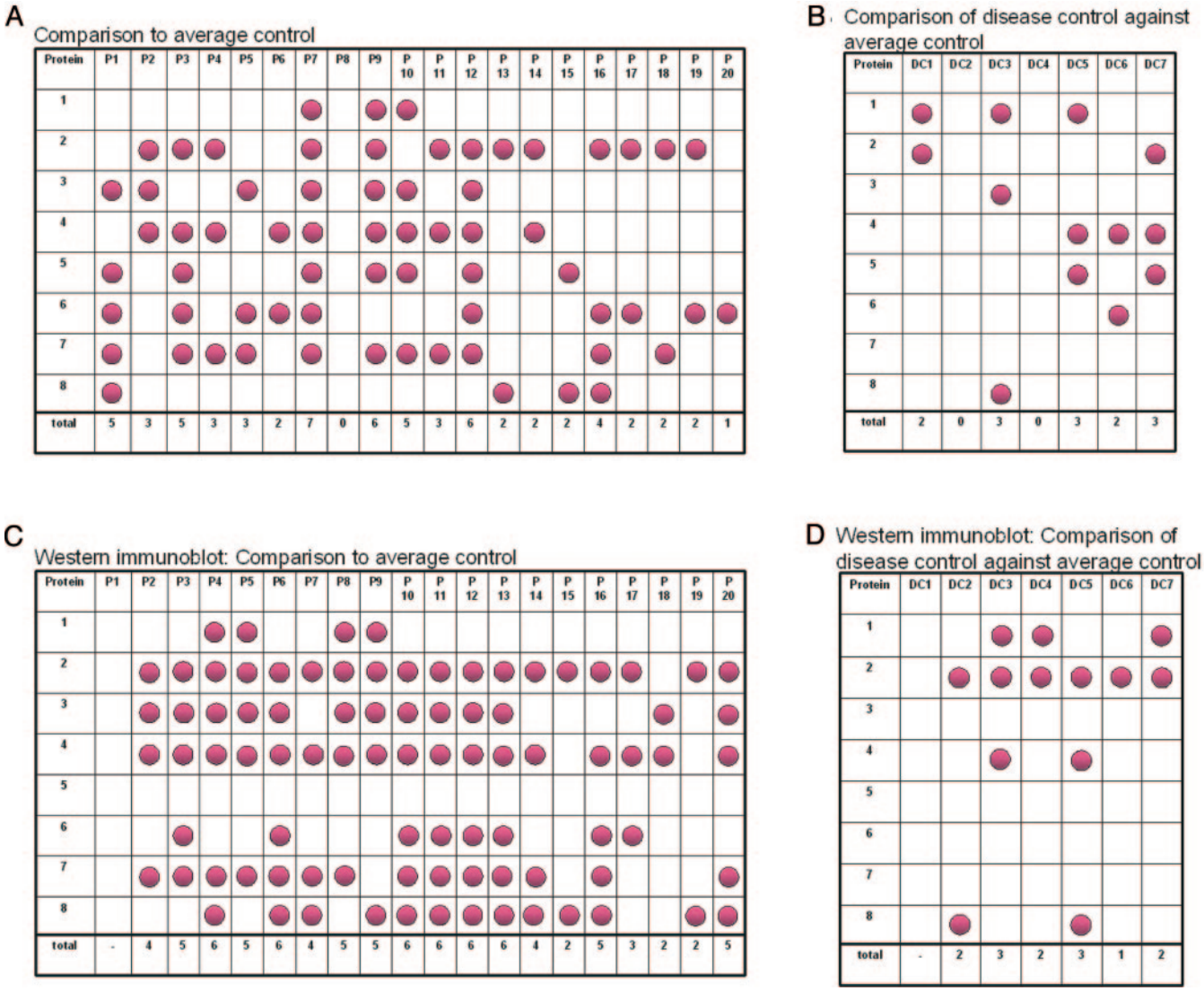

FIG. 3. **Identification of alopecia areata patients by detection of two or more autoantigens.** *A*, comparison of each patient's signal intensity to the average intensity of the control group. Autoantigens that show a double or higher intensity are marked with a *red circle*. *B*, comparison of each disease control signal intensity to the average intensity of the control group. Autoantigens that show a double or higher intensity are marked with a *red circle*. *C*, detection of putative autoantigens on Western blots with patient sera. Specific signals for autoantigens were detected on a FujiFilm LAS-1000 imager after background deduction relative to human IgG input. The figure summarizes the results of three independent blots. *D*, Western blot analysis of the putative autoantigens with sera from disease patients. Detection was performed as described in *C*. *Protein 1*, SCG 10; *2*, GLCDAC05; *3*, α-endosulfine; *4*, NOL8; *5*, FGFR3; *6*, dematin; *7*, signal recognition particle subunit 14; *8*, EPF autoantigen.

istic for each disease. Alternatively the relevance criteria should be chosen more stringently. However, this also requires an improvement of the disease-associated protein chip. We suggest that a protein biochip leading to an average detection of about five autoantigens may result in a better discrimination of related diseases. A further selection of putative autoantigens can be achieved using for example another type of expression library such as a T-cell-specific or a tissue-specific expression library.

Functional Relation of Putative Autoantigens—We identified eight putative autoantigens by profiling the autoantibody repertoire of alopecia areata patients (Table I). We detected proteins with no clear pathophysiological role except FGFR3, which reveals a strong relation to hair diseases. Below we discuss the role of some of the identified proteins.

FGFR3 shows homology to epidermal growth factor receptor, which plays an important role in the control of hair cycle progression. Expression of FGFR3 was strongly detected in the superbasal layers and the inner layers of hair follicles and in wounded skin. Using RNA *in situ* hybridization analysis, FGFR3 RNA was detected in precuticle cells in the periphery of the hair bulb. However, the function of

FGFR3 in the autoimmune disease alopecia areata is not clear.

The endemic pemphigus foliaceus (EPF) autoantigen originally was identified by screening a keratinocyte expression library with autoantibodies from a patient with the blistering skin disease EPF. The isolated cDNA encodes a member of the ubiquitin carrier proteins (E2). Interestingly this autoantigen is generated by a translational reading frameshift from the same cDNA (26, 27). The relevance of this autoantigen in the pathogenesis of EPF or of other autoimmune diseases is currently unknown.

Dematin is an actin-binding and bundling protein of the erythrocyte membrane skeleton. SCG10-related proteins have a function in microtubule destabilization. The functional block by phosphorylation further supports the importance of the SCG10 family proteins in neuronal cytoskeletal regulation, particularly as to microtubule dynamics.

However, it is unclear whether the identified potential autoantigens are functionally related to the autoimmune disease. When comparing various putative autoantigens specific for other autoimmune diseases, such as rheumatoid arthritis, only some autoantigens are described with known functional significance in the disease. For other diseases no functional significance was found for these autoantigens (28). Interestingly, however, some of those autoantigens with unknown function in relation to the autoimmune disease have still been used as biomarkers of the corresponding disease (29).

In an another approach, we have profiled the autoantibody repertoire of an SLE mouse model on a mouse TH_1 cDNA expression library and identified for example the mouse lectin and galactose-binding protein soluble 3 protein as a putative autoantigen (30). When compared with the human SLE, autoantibodies against the human lectin and galactoside-binding soluble 3 (galectin-3) have been described already, indicating a certain homology between mouse models to human diseases (31). This suggests that applying our strategy to the research of autoimmune diseases leads to valuable data.

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