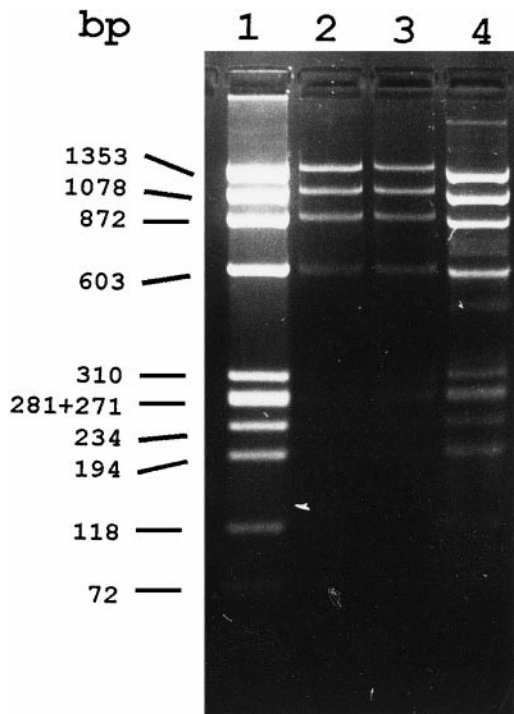

FIG. 1. Comparison of yields of DNA fragments isolated by DEAE paper and several other methods. One microgram of the ΦX174 DNA cut with *Hae*III was loaded into three lanes of 1.4% agarose gel in TAE with 0.5 μg/ml ethidium bromide (1). After a short electrophoresis (DNA samples just entered the gel, bromphenol blue was about 5 mm from the slots), the pieces of the gel containing all DNA fragments from two lanes were removed, and DNA was isolated by commercial kits for isolating DNA fragments from agarose gels. The third lane was used for isolating DNA fragments by DEAE paper. A slot was cut in front of the DNA fragments in gel, a piece of DEAE paper was inserted into the slot, and electrophoresis was continued for 30 min, until all the fragments were bound on the DEAE paper. The fragments were isolated from the DEAE paper as described in the text. The fragments isolated by the particular methods were electrophoresed on the 2% agarose gel. Lanes: 1, 1 μg of the ΦX174 DNA cut with *Hae*III; 2, the DNA fragments isolated by QIAquick column (Qiagen); 3, the modification of the GeneClean technique (2); 4, the DNA fragments isolated by DEAE paper.

glass plate) was covered by Saran wrap, labeled by hot ink, and exposed 10 min to autoradiography film. The region corresponding to the fragment of interest was cut with a scalpel, the Saran wrap was removed, and the sandwich with the piece of the DEAE paper and Whatman 3MM paper was prepared as for unlabeled DNA fragment. The rest of the procedure was identical to that for the unlabeled DNA fragment.

The method described for isolation of DNA fragments from both agarose and polyacrylamide gels is efficient, simple, and cheap. The yield of the method was about 80–90% as measured after isolation of ^{32}P-labeled DNA fragments from 100 bp to 4 kb. We have found a similar yield using low-melting-point agarose. The fragments could be used for ligation, digestion with restriction enzymes, 5′-, 3′-end labeling with T4 polynucleotide kinase or Klenow fragments of DNA polymerase I, respectively, or random-primed labeling. The glycogen present in the fragment did not interfere with any reaction used. A comparison of the method with the two commercial techniques (GeneClean and QIAquick) is exemplified in Fig. 1.

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Limitations of Comparative Detection of Proteins via Epitope Tagging

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Epitope tagging affords a powerful means by which to facilitate purification, manipulation, and detection of recombinant proteins. This technique involves the introduction of a short specific sequence at any point within the coding region of the protein of interest, which can later be recognized by an antibody raised against the introduced epitope. Epitope tags are generally small enough so as not to affect the biological activity of the protein and can allow multiple protein species to be detected with the same antibody. An extension of this application is to assess comparative expression levels through detection of the same tag

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sequence on different proteins. Although this approach affords a means by which to compare distinct proteins with a single antibody, it assumes that recognition of the tag occurs with equal efficiency in different molecular contexts. We show here an example in which the recognition of an epitope tag occurs with very different efficiency between two molecules, and suggest that this technique may have serious limitations.

The original goal of our studies was to determine the stoichiometry of a complex formed between the desmosomal cadherin molecule, desmoglein 1 (Dsg1),[2] and its catenin binding partner, plakoglobin (Pg). To facilitate direct quantitation of proteins in the complex, we attached to the carboxyl terminus of each of these molecules a 10-amino acid segment (EQKLISEEDL) of the human c-myc proto-oncogene. The myc sequence is one of the most commonly used epitope tags and is efficiently detected by the monoclonal antibody 9E10 (1). Our results suggest that the tag is not equally accessible to 9E10 recognition in the context of the C-terminus of Dsg1 vs the C-terminus of Pg.

Materials and methods. Mouse L-cell fibroblasts stably expressing human desmoglein 1 · myc (Dsg1) and human plakoglobin · myc (Pg), or an empty neomycin resistance vector were generated by calcium phosphate transfection and subsequent selection in G418 (Geneticin) (Cellgro, Herndon, VA) as previously described (2). ^{35}S metabolic labeling (3 h) and immunoprecipitation (in Tris-buffered saline containing 1% Triton X-100) were carried out as previously described (3). Complexes were immunoprecipitated with 3 μl 982 human pemphigus autoimmune serum ((gift from Dr. J. Stanley) against the extracellular domain of Dsg1). Immobilized proteins were exposed to a Fuji phosphorimaging plate (Fuji Medical Systems, Stamford, CT) and data were captured on a STORM imager (Molecular Dynamics, Sunnyvale, CA). Western blotting of the same nitrocellulose membrane was carried out as previously described (3), with 9E10 anti-myc monoclonal antibody. Enhanced chemiluminescence was performed as previously described (3). The blot was exposed to X-ray film (Fuji Medical Systems). Densitometric analysis of both the ^{35}S and chemiluminescent signals was carried out with NIH Scion Image software (Scion Corp., Frederick, MD).

Recombinant Dsg1 cytoplasmic tail (his · Dsg · myc) was generated by PCR amplification and subsequent insertion of nucleotides 1921–3360 of the human Dsg1 cDNA (GenBank Accession No. AF097935) plus the myc tag sequence into pQE-32 (Qiagen, Chatsworth, CA). This construct produces a fusion protein comprising 6 N-terminal histidine residues followed by the intracellular domain of Dsg1 and a C-terminal myc tag.

[2] Abbreviations used: Dsg1, desmoglein 1; Pg, plakoglobin.

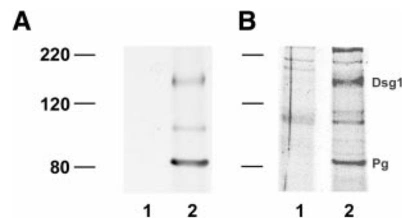

FIG. 1. Western blot with anti-myc antibody does not detect all immobilized myc-tagged proteins with equal efficiency. L-cell fibroblasts stably expressing myc-tagged desmoglein 1 (165 kDa) and myc-tagged plakoglobin were metabolically labeled for 3 h, lysed, and subjected to immunoprecipitation with a human anti-Dsg1 antiserum and resultant immobilized proteins were exposed to film (B). The same nitrocellulose page was later processed for immunoblot with 9E10 monoclonal anti-myc antibody (A). Quantitation of data was carried out by densitometric analysis of scanned images using Scion Image software. After correction for labeling based on amino acid composition, the Pg:Dsg ratio revealed by the radioactive signal was found to be 0.9:1, whereas the chemiluminescent signal was found to be 5:1, indicating a difference in immunodetectability of the myc tag between the two proteins. In each panel, lanes 1 and 2 refer to Neo control lysate or Dsg1/Pg line, respectively.

DH5α *E. coli* expressing his · Dsg · myc were grown to optimal density and protein was purified over nickel–NTA agarose (Qiagen) according to manufacturer's instructions. Recombinant plakoglobin (pMALPg · myc) was generated by inserting nucleotides 120–2351 plus the myc tag sequence into the pMALC2 vector (NEB, Beverly, MA). This construct produces a fusion comprising maltose-binding protein followed by full-length human plakoglobin and a C-terminal myc tag. DH5α *E. coli* expressing pMALPg · myc were grown to optimal density and protein was purified over maltose according to manufacturer's instructions (NEB) and dialyzed into PBS.

Proteins were separated by 7.5% SDS–PAGE and either stained with SYPRO ruby protein gel stain (Molecular Probes, Eugene, OR) according to manufacturer's instructions or transferred to nitrocellulose and immunoblotted with 9E10 anti-myc antibody as previously described. SYPRO-stained gels were visualized by UV transillumination and photographed, or scanned on the STORM imaging system; Western blots were processed as previously described, exposed to film and all densitometric analysis was carried out with NIH Scion Image software (Scion Corp.).

Results and discussion. To determine the stoichiometric relationship between Dsg1 and its known binding partner Pg, we coimmunoprecipitated Dsg1/Pg complexes from metabolically labeled stable L-cell fibroblast lines. Immunoprecipitation was carried out with an antibody against the extracellular domain of Dsg1. A semiquantitative immunoblot of immobilized protein with a 9E10 antibody recognizing the myc-epitope tag on each protein suggested a Pg:Dsg1 stoichiometry of approximately 5:1 (Fig. 1A). However,

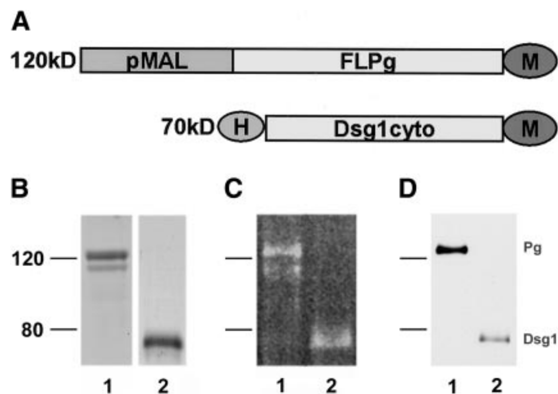

FIG. 2. Analysis of recombinant myc-tagged plakoglobin and desmoglein 1 verifies a quantifiable difference in detectability of the myc tag. One set from a series of dilutions of purified pMalPg · myc (120 kDa total) and purified his · Dsg · myc (70 kDa total) (A)is shown here, which were visualized by Coomassie stain (B), or stained with SYPRO ruby stain (C), and photographed. (Note: purified Pg includes a smaller breakdown product, which is approximately 20 kDa shorter than the full-length molecule, and does not include the myc-tagged C-terminus (data not shown); therefore, only the higher band was included in Pg quantitation.) 1:10 dilutions of the same samples were subjected to immunoblotting (D). Scanning densitometry and comparison of stained gels and Western blots indicates a fivefold difference in the detectability of the myc tag on Pg vs the myc tag on Dsg1. In B, C, and D, lanes 1 and 2 refer to recombinant Pg or Dsg1 tail, respectively.

prior exposure of the same blot to X-ray film for 3 h to capture the ^{35}S signal indicated a Pg:Dsg1 ratio of 0.75:1 (Fig. 1B). Correction for labeling based on the amino acid composition of the two proteins suggests an actual ratio of approximately 0.9:1, and duration of labeling had been determined to be sufficient to achieve steady state (data not shown). Several exposure lengths generated the same stoichiometric ratios, indicating that detection of the radioactive signal was within linear range. This clear discrepancy between data collected by the two independent means suggested that the antibody might be recognizing the epitope tag on immobilized Dsg1 · myc and immobilized Pg · myc differently. A similar discrepancy was observed in each of four independently derived Dsg1/Pg stable L-cell lines (data not shown). To confirm the observation, an alternative means of direct quantitation of the level of immobilized protein of each species was necessary. To this end, we generated recombinant myc-tagged plakoglobin and myc-tagged Dsg1 cytoplasmic tail (Fig. 2A). Serial dilutions of these proteins were separated by SDS–PAGE and either visualized by Coomassie stain (Fig. 2B) or stained with SYPRO ruby protein stain, which binds nonspecifically to the SDS coat of electrophoresed proteins (Fig. 2C) and is quantitative over three orders of magnitude (4). Subsequent quantitation of SYPRO signal was carried out by densitometric analysis of captured images of the SYPRO-stained gel, and was verified by Bradford assay (5) of protein stocks. In parallel, 1:10 dilutions of the same protein samples were run on a second gel and subjected to Western blotting with 9E10 antibody (Fig. 2D). Densitometric analysis of the SYPRO-stained gel, and the Western blot indicated Pg:Dsg ratios of 1:1.2 and 4.5:1, respectively. Therefore, after correction for the amount of protein loaded, it appears that the myc tag on Pg is recognized with an efficiency approximately 5× that of the same tag on Dsg1. DNA sequence analysis of the tag in each construct revealed no mutations which could give rise to such poor antibody recognition.

Previous literature has established that higher order structure of a protein affects the antigenic specificity of small peptides within the structure (6). Additionally, the conformation of the immunogen is an important element of its antigenicity (7), and residues adjacent to a peptide sequence may play an important role in determining reactive discrimination by an antibody (8). As small epitope tag-recognition is a commonly applied approach to quantitative analysis of protein species, we sought to test its accuracy. Through direct, quantitative assessment of immobilized polypeptides, followed by immunoblot with the anti-tag antibody, we demonstrate a difference in the immunoreactivity of the myc peptide sequence when attached to unlike proteins. These data extend an earlier observation that some myc-tagged protein species could not be recognized by 9E10 immunoblot despite verification of their presence with antibodies specific to the proteins themselves (9). Together, these results suggest that recognition of small epitopes may be dependent on contextual requirements of folding in the vicinity of the tag and that these requirements apply also to the immunorecognition of denatured, immobilized proteins. We conclude that comparison based on tag-recognition is subject to severe limitations and may be misleading when used as a means to assess distinct proteins.

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Site-Specific Anti-C3a Receptor Single-Chain Antibodies Selected by Differential Panning on Cellulose Sheets

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Different procedures have been described to select antigen-specific antibodies from combinatorial antibody libraries by means of phage-display technology [reviewed in (1–3)]. We previously selected a panel of scFv fragments, with specificity for the second extracellular loop of the human C3a receptor (2e loop), from combinatorial phage-display libraries, generated from immunized mice using purified antigen coupled to immunotubes [C3aR-library (4)]. The selected scFvs could be assigned into three different groups, represented by the scFvs 3G7, 2A1, and 3C6, according to DNA sequence homologies. Two different epitopes within the 2e loop were recognized, i.e., clones 3G7 and 3C6 bound to PDFYGDPLE (amino acids 185–193 of the C3a receptor) and clone 2A1 bound to TNDHPWTVP (amino acids 218–226), which were then defined as the immunodominant epitopes of the 2e loop.

Methods

Preparation of cellulose sheets (Cs).[3] Fifty 15mer peptides with an offset of 3 amino acids, spanning the whole 2e loop from amino acids 172 to 332 were produced by the spot synthesis technique and immobilized by β-Ala–β-Ala dipeptide anchors (5 nmol peptide per spot) on cellulose sheets (1.0 × 6 cm, Whatman 540), using an ASP 222 spotting robot as previously described (11).

Selection on Cs. Phage from the initial library and from subsequent selection rounds were prepared exactly as described (4). For each panning round, Cs was blocked with PBS containing 2% low-fat dry milk (MPBS) for 2 h at 37°C and 2×10^{12} phage were incubated in a total volume of 500 μl MPBS for 2 h with gentle shaking. After washing Cs 10 times with PBS/Tween 20 (0.1%) and 10 times with PBS, bound phage were eluted by a 10-min incubation of 3 ml HCl/glycine (0.1 M, pH 2.2, supplemented with 1% bovine serum albumin), subsequently neutralized with 300 μl Tris/HCl (1 M, pH 9.1), and rescued as described (4).

Phage ELISA. Single-phage clones were tested for antigen binding by ELISA exactly as described (4), using the purified 2e loop as antigen.

Epitope mapping. The epitopes of scFvs were determined, using scFv containing bacterial culture supernatant, as described (4).

FACS analysis. ScFv binding to the native C3a receptor was assessed in FACS analysis as previously described (4).

Results and Discussion

Our intention was to isolate antibodies from the C3aR library (4), with specificity other than toward the immunodominant regions. Therefore, we developed a selection method, which we call "differential panning," based on peptides fixed to Cs.

First, we examined whether panning on Cs would result in the selection of the same antibodies, compared to panning in immunotubes (4); thus, panning

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[3] Abbreviations used: Cs, cellulose sheets; PBS, phosphate-buffered saline; MPBS, PBS containing 2% low-fat dry milk; ELISA, enzyme-linked immunosorbent assay.

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