RasGAP-like Protein IQGAP1 is Expressed by Human Keratinocytes and Recognized by Autoantibodies in Association with Bullous Skin Disease

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Autoantibodies in patients with autoimmune bullous skin diseases, such as pemphigus or bullous pemphigoid are of diagnostic value and might play a part in the pathogenic scenario. In this study we present five patients with erythematous plaques, subepidermal blister formation of the skin, and the presence of circulating autoantibodies directed against a so far unrecognized 190 kDa antigen in human keratinocytes. Amino acid sequence analysis identified the protein as IQGAP1, a recently described human Ras GTPase-activating-like protein suspected to act as an effector molecule for Cdc42 and Rac1, members of the Rho small GTPase family and to play a key part in regulating E-cadherin-mediated cell adhesion. The protein is selectively recognized by a monoclonal anti-IQGAP1 antibody on western blots and immunoprecipitates from keratinocyte extracts. Indirect immunofluorescence locates IQGAP1 within individual keratinocytes in a cytoplasmic pattern and along the cell periphery at adhesive sites. Our results demonstrate IQGAP1, a newly described multifunctional protein, to be constitutively expressed in human keratinocytes where it may contribute to the integrity of the epidermal layer. Furthermore, we found autoantibodies reacting with IQGAP1 in patients with bullous skin eruptions most apparently belonging to the spectrum of bullous pemphigoid. ***Key words: autoimmunity/cellular structure/epidermal proteins/subepidermal blistering. J Invest Dermatol 120:365–371, 2003***

Autoimmune bullous skin diseases, such as disorders of the pemphigus group or bullous pemphigoid (BP) are defined by typical clinical presentation, distinct histologic and immunomorphologic changes of lesional or perilesional skin, and by the presence of serum autoantibodies directed against specific epidermal antigens (Amagai *et al*, 1991; Stanley, 1993; Nousari and Anhalt, 1999; Anhalt and Diaz, 2001). Several studies have revealed important components of the cellular and humoral autoreactive pathologic events in these autoimmune bullous skin diseases, including the antigens involved (Anhalt *et al*, 1990; Koch *et al*, 1997; Amagai *et al*, 1998, 2000; Lin *et al*, 2000), the associated major histocompatibility complex class II genes (Sinha *et al*, 1988), and the direct effect of antibody–antigen binding, e.g., in pemphigus vulgaris or paraneoplastic pemphigus by passive transfer of serum autoantibodies into newborn mice (Anhalt *et al*, 1982, 1990). Patients with pemphigus vulgaris or pemphigus foliaceus develop lesions of the skin and mucous membranes exhibiting loss of cell–cell adhesion among keratinocytes resulting in intraepidermal blister formation (Amagai *et al*, 1991; Stanley, 1993; Nousari and Anhalt, 1999; Anhalt and Diaz, 2001). These disorders are characterized by the presence of *in vivo* bound and circulating autoantibodies directed against desmoglein 1 and/or desmoglein 3, members of the cadherin family mediating cell–cell adhesion (Amagai *et al*, 1991; Buxton *et al*, 1993; Stanley, 1993; Anhalt and Diaz, 2001). In the more recent past, paraneoplastic pemphigus has been described in patients with underlying neoplasms developing severe erosions of the mucous membranes, erythema multiforme-like or lichenoid skin eruptions, and associated lethal pulmonary involvement (Anhalt *et al*, 1990; Nousari and Anhalt, 1999). The heterogeneous clinical features of paraneoplastic pemphigus are coexistent with the presence of distinctive serum autoantibodies directed against a group of antigens, including desmoglein 1 and 3 and proteins of the plakin group as there are desmoplakin I and II, BP antigen (BPAG) 1, periplakin, envoplakin, and plectin (Anhalt *et al*, 1990; Amagai *et al*, 1998; Kiyokawa *et al*, 1998; Proby *et al*, 1999). Autoantibodies directed against desmoplakin I and II have further been shown in a severe subset of erythema multiforme, a syndrome of inflammatory skin eruptions (Huff *et al*, 1983) with erythematous iris-like plaques, blistering, and involvement of the mucous membranes but without underlying neoplasms; this indicates that in addition to T cell-mediated pathophysiologic events operative in erythema multiforme, humoral autoimmunity against the desmoplakins may play a crucial part in this disease subset (Foedinger *et al*, 1995, 1998). Autoantibodies targeting molecules of the dermoepidermal basement membrane zone are found in BP associated with subepidermal bulla formation (Mutasim *et al*, 1989; Stanley, 1993; Nousari and Anhalt, 1999). BPAG 1, known as a member of the plakin family

Manuscript received March 17, 2002; revised September 16, 2002; accepted for publication November 14, 2002

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Abbreviations: BPAG, bullous pemphigoid antigen; GAP, GTPase-activating protein.

0022-202X/03/$15.00 ·

of proteins (Muller *et al*, 1989; Ruhrberg and Watt, 1997), is found at the cytoplasmic plaque of hemidesmosomes, whereas BPAG 2 also designated type XVII collagen is a transmembrane protein with collagenous repeats in its extracellular domain (Areida *et al*, 2001).

In this study, we show expression of the human RasGAP(GTPase-activating protein)-like protein IQGAP1 (Weissbach *et al*, 1994) in human keratinocytes. IQGAP1 is a recently described RasGAP-related protein that acts as an effector molecule for Cdc42 and Rac1, members of the Rho small GTPase family, to mediate cellular processes involving calmodulin (Weissbach *et al*, 1994). It is thought to play a key part in regulating E-cadherin mediated cell–cell adhesion and cytoskeletal organization (Kuroda *et al*, 1998). Additionally, we report for the first time, IQGAP1 as antigenic target for circulating autoantibodies in patients with distinct bullous skin eruptions.

MATERIALS AND METHODS

Patients Two hundred and five sera of patients with pemphigus vulgaris (n = 30), pemphigus foliaceus (n = 5), erythema multiforme (n = 80), and BP (n = 90) were investigated for the presence of circulating autoantibodies directed against epidermal antigens. Five patients with anti-190 kDa autoantibodies (age 31–88, two male and three female) exhibited disseminated plaques and bullous skin eruptions localized predominantly on acral sites. The skin lesions varied in number and size, with intact small blisters or larger bullae, urticarial or targetoid plaques with and without erosions. Two of them (patients 1 and 2) showed mild involvement of the oral mucous membranes. Clinical diagnoses were bullous erythema multiforme (patient 1), erythema multiforme-like eruption (patients 2 and 3), and BP (patients 4 and 5). Skin lesions of all patients cleared under systemic treatment with steroids, and, recurred infrequently upon cessation of therapy. Patient 1 presented a 10 y history of recurrent erythema multiforme obviously associated with herpes simplex infection and had, in addition, a *Helicobacter pylori*-positive gastritis. Patient 2 was suffering from colitis ulcerosa and patient 4 had a history of colon carcinoma treated surgically 7 y before the outbreak of the bullous disease. Screening of all patients by laboratory parameters, X-ray and computed tomography was negative for underlying neoplasia at the time of observation.

Tissue, sera, and antibodies Biopsy specimens of skin lesions were processed for routine histology and for direct immunofluorescence as described (Foedinger *et al*, 1998). Samples for immunofluorescence are placed in RPMI 1640 media (Gibco, Paisley, Scotland, U.K.) for transport. Sections of monkey esophagus were purchased from INOVA (San Diego, CA). Normal human skin was obtained from healthy volunteers and processed for indirect immunofluorescence and split skin diagnosis according to methods routinely used in our laboratory. Shortly, normal human skin was exposed to 1 mM NaCl for 48–72 h to form split skin and subsequently snap frozen in liquid nitrogen. Cryosections (4 μm) were prepared from split skin samples as well as snap frozen normal human skin. Serum samples from the five patients described above were used to perform experiments to assess the specificity of the 190 kDa antigen. For control experiments, sera of patients with paraneoplastic pemphigus (n = 2), BP (n = 6), and from healthy volunteers (n = 10) were used.

Mouse monoclonal anti-IQGAP1 antibody (clone 24) and polyclonal rabbit anti-IQGAP1 antibody (raised against a recombinant protein corresponding to amino acids 314–422 of IQGAP1 from human origin) were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Mouse monoclonal antibody to desmoglein (clone dg 3.10) was from Progen (Heidelberg, Germany). For control immunoprecipitation we used an isotype matched murine antibody (Pharmingen, San Diego, CA). As second step reagents for immunofluorescence we applied fluorescein isothiocyanate conjugated goat anti-human C3 (INCSTAR Corporation, Stillwater, MN), affinity-purified FITC-conjugated goat $F(ab')_2$ anti-human IgG (Jaxell, San Diego, CA), TRITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Jaxell), and for immunoblotting experiments, alkaline phosphatase-conjugated goat anti-human, goat anti-mouse IgG, and goat anti-rabbit IgG (Promega Corp., Madison, WI).

Immunofluorescence experiments Cryosections of normal human and split skin samples as well as monkey esophagus were incubated with serum samples of interest, diluted 1 : 40 in phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) (Sigma, St Louis, MO). For detection of bound antibodies FITC-conjugated goat $F(ab')_2$ anti-human IgG was used diluted 1 : 100 in PBS/0.1% BSA. For direct immunofluorescence, 4 μm cryosections of skin samples were prepared, preincubated with PBS/1% BSA, and exposed to FITC-conjugated goat $F(ab')_2$ anti-human IgG (Jaxell) or FITC-conjugated goat anti-human C3 antibody (INCstar Corporation). For indirect immunofluorescence on cultured keratinocytes, normal human keratinocytes obtained from surgically removed skin were grown to confluence in chamber slides (Falcon, Becton Dickinson, Franklin Lakes, NJ) using keratinocyte growth medium (Clonetics Corp., San Diego, CA). Cells were fixed in 3% paraformaldehyde in PBS, washed with PBS/0.1% BSA, permeabilized with PBS containing 0.1% Triton-X 100 for 10 min, and incubated with a mixture of serum sample from the respective patient and the monoclonal anti-IQGAP1 antibody, diluted 1 : 40 in PBS/0.1% BSA, washed, and subsequently incubated with the FITC-conjugated goat $F(ab')_2$ anti-human IgG (Jaxell) and TRITC-conjugated goat anti-mouse IgG (Jaxell). After final washing, cells and sections were embedded in Fluoprep (BioMerieux, Marcy lÈtoile, France), coverslipped and examined in a fluorescence microscope (Olympus BH-2, Melville, NY).

Immunoblotting and immunoprecipitation Biochemical procedures were performed according to methods described previously (Foedinger *et al*, 1995). Normal human keratinocytes obtained from surgically removed skin grown to near confluence were scraped and homogenized in 1% nonidet P-40 (NP-40) (Calbiochem-Novabiochem, La Jolla, CA)/Tris-buffered saline, pH 7.4, with 2 mM phenylmethylsulfonyl fluoride (Sigma) and a cocktail of proteinase inhibitors at a concentration of 5 mM (leupeptin, antipain, chymostatin, pepstatin, all Calbiochem). Proteins were precipitated by incubation with ethanol–acetone 1 : 1 solution overnight at −20°C, and pellets resolubilized in 10% sodium dodecyl sulfate (SDS) sample buffer. Extracted proteins were loaded on to 5% SDS slab gels for polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Strips of the blotted epidermal proteins were blocked, incubated with patients' sera or the respective controls, washed, exposed to alkaline phosphatase-conjugated second step antibody (Promega) followed by nitrobluetetrazolium/bromo-chloroindolyl phosphatase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MA) to visualize bound antibodies. Alternatively, epidermal sheets were used for protein extraction and processed the same way.

For immunoprecipitation, 1 ml aliquots of NP-40 lysates of cultured keratinocytes or NP-40 Tris-buffered saline were incubated with 10 μg mouse monoclonal anti-IQGAP1 antibody (Transduction Laboratories), for 1 h, at 4°C. Antigen–antibody complexes were precipitated with staphylococcal protein G-Sepharose (Sigma), boiled for 3 min in 10% SDS sample buffer and separated by SDS–PAGE on 5% gels. Separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), strips were cut out and further processed for immunoblotting with patients' sera and several controls. Alternatively, confluent cultured keratinocytes were incubated overnight with C-14-labeled amino acids (New England Nuclear Boston, MA). NP-40 extracts of cells were prepared, preabsorbed with normal human serum and then incubated with sera of patients recognizing IQGAP1, normal human sera, mouse monoclonal anti-IQGAP1, and polyclonal anti-IQGAP1 antibody. The precipitated antigens were separated by SDS–PAGE on 5% gels and transferred to nitrocellulose. Thereafter, the membrane was exposed to mouse monoclonal anti-IQGAP1, and bound antibodies were visualized by alkaline phosphatase-conjugated anti-mouse IgG and the respective enzyme reaction. Additionally, the membrane was exposed for 6 wk to visualize precipitated antigens by autoradiography.

Protein identification Lysates of cultured human keratinocytes were loaded on to 5% SDS–PAGE slab gels and separated electrophoretically. One-third of the gel was cut and proteins blotted to nitrocellulose. Strips immunoblotted with serum of one patient were used to locate the protein band of interest. The remaining parts of the gel were stained with Coomassie Serva Blue G (Heidelberg, Germany). The corresponding protein band was cut out of the gels and frozen in liquid nitrogen. Tryptic digestion, mass spectrum analysis of the generated peptides, and database analysis (SWISS-PROT database; Bairoch and Apweiler, 2000) for protein identification was performed by Protana (Odense, Denmark).

BP180 enzyme-linked immunosorbent assay (ELISA) In order to test sera of patients for the presence of autoantibodies directed against BPAG 2 (180 kDa) we performed an ELISA using the BP180 ELISA kit (MBL Medical & Biological Laboratories, Naka-ku Nagoya, Japan). The ELISA is based on the recombinant BP180NC16a protein used as solid phase antigen. We examined the sera of patients 1–5, sera of BP control

patients as well as normal human sera according to the manufacturer's instructions (MBL). Briefly, calibrator and patients' sera are added to the microwell coated with the BP180 antigen. After washing and application of the peroxidase conjugate, the substrate incubation induces enzyme reaction and color development. Adsorbance is photometrically evaluated at 450 nm. The unit value (anti-BP180 value) is calculated as given in the manufacturer's manual and is seen positive when equal or larger than 9.

RESULTS

Demonstration of histology and *in vivo* bound immunoreactants Histologic investigation of biopsy samples of skin lesions (**Fig 1*a***) showing lesion of patient 4 diagnosed as BP) revealed subepidermal blistering, spongiosis, and dermal inflammatory infiltrate with neutrophils and eosinophils (**Fig 1*b***). In addition, single keratinocytes of the basal cell layer even gave the impression of focal detachment from neighboring cells (**Fig 1*b***). Direct immunofluorescence of perilesional skin exhibited discrete dots of bound IgG (**Fig 1*c***) concentrated at adhesive sites of neighboring keratinocytes. The labeling could be seen along the cell periphery of individual keratinocytes throughout the epidermis, whereas the most intense labeling was found at the basal and suprabasal cell layers. Direct immunofluorescence from patient 5 showed additional linear IgG along the basement membrane zone (not shown). C3 was found in a linear pattern along the basement membrane zone in all biopsy specimens (**Fig 1*d***).

Circulating serum autoantibodies recognize a 190 kDa antigen of keratinocytes Using immunofluorescence and immunoblotting to search for the presence of circulating autoantibodies in five of 205 sera of patients with various bullous skin eruptions we found a new, so far unknown 190 kDa antigen recognized by autoantibodies. When we tested serum samples of the five patients by indirect immunofluorescence on monkey esophagus we found cytoplasmic staining of basal and suprabasal cells with bound IgG predominantly at the cell periphery (**Fig 2*a***). Serum of patient 5 exhibited additional linear staining along the basement membrane zone (**Fig 2*b***); however, the latter reaction pattern cannot necessarily claim specificity as 8% of our BP sera (n = 90) gave a similar reaction. Split skin analysis revealed linear staining of the blister roof only with serum of patient 5 (**Fig 2*d***). Immunoblotting NP-40 lysates of cultured human keratinocytes with sera of the five patients diagnosed as erythema multiforme (patient 1) or erythema multiforme-like eruption (patients 2 and 3) and BP (patients 4 and 5) showed binding of autoantibodies to a polypeptide at 190 kDa (**Fig 3**, lanes 5–9). Immunoblot of the serum from patient 5 showed an additional band at 230 kDa (**Fig 3**, lane 5). Immunoblotting with serum samples of further BP patients showed reactivity at 230 kDa (BPAG 1) and 180 kDa (BPAG 2) (**Fig 3**, lane 2). Periplakin, the 195 kDa antigen recognized by paraneoplastic pemphigus serum (**Fig 3**, lanes 3 and 4) was found clearly above the respective 190 kDa polypeptide. The sera of the five patients obviously recognized a new, so far unidentified antigen.

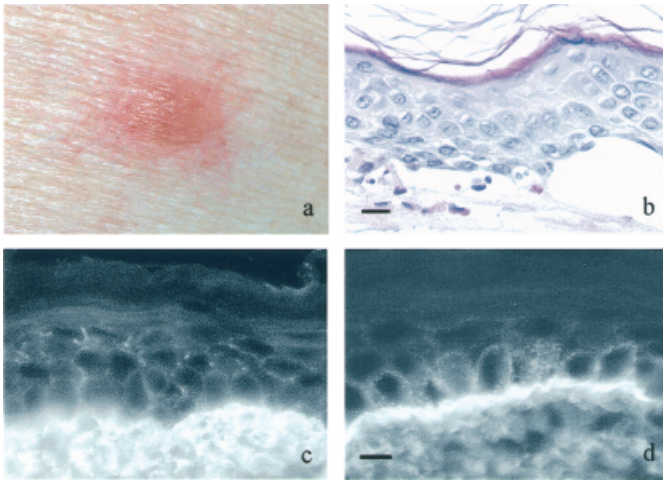

Figure 1. A bullous lesion of patient 4 and its histologic and immunomorphologic appearance. (*a*) The bulla on erythematous skin of patient 4 diagnosed as BP exhibits (*b*) subepidermal blistering, dermal eosinophils, and, single keratinocytes detaching from the neighboring cells in the basal cell layer. (*c*) Direct immunofluorescence from the same lesion reveals bound IgG in a dotted pattern along the cell periphery of individual keratinocytes and (*d*) C3 along the basement membrane zone. *Scale bars*: 20 μm.

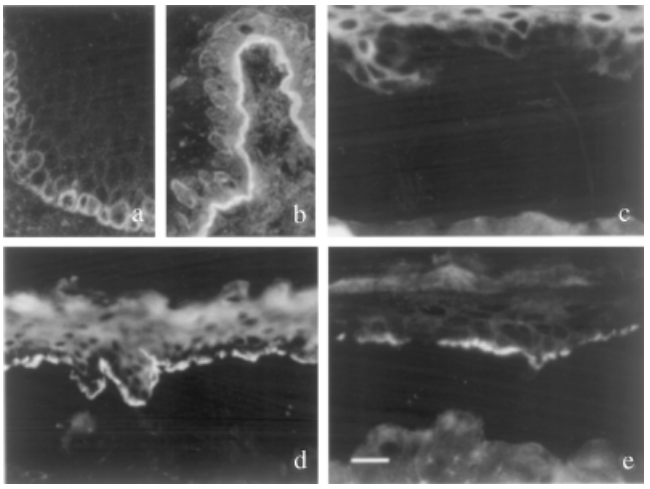

Figure 2. Indirect immunofluorescence suggests the presence of circulating autoantibodies against epidermal antigens. (*a*) Serum sample from patient 1 gives cytoplasmic staining at the cell periphery of the basal cells, whereas (*b*) serum from patient 5 reveals additional linear IgG along the basement membrane zone of monkey esophagus. Indirect immunofluorescence on normal human NaCl split skin performed with serum samples from patients 1–4 reveals cytoplasmic staining within the epidermis and no labeling of the basement membrane zone (*c*, patient 4), whereas (*d*) serum of patient 5 shows linear staining of the blister roof. Serum of a BP patient gives linear staining along the blister roof in split skin analysis (*e*). *Scale bar*: 40 μm.

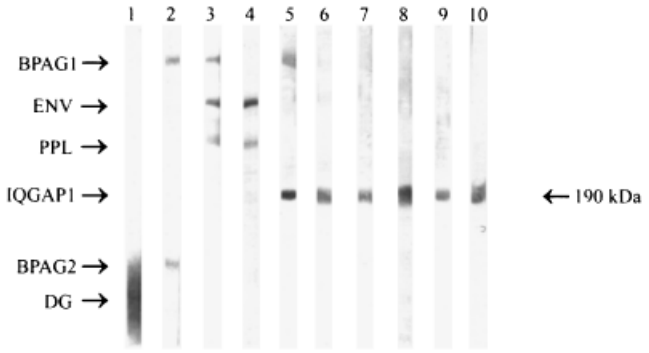

Figure 3. Immunoblotting on epidermal extracts reveals reactivity at 190 kDa. *Lanes 5–9* show immunoblot from patients 1 to 5 exhibiting the distinct band at 190 kDa, patient 5 (*lane 5*) additionally recognizing a protein at 230 kDa appearing as BPAG 1. *Lane 2* shows BPAG 1 (230 kDa) and BPAG 2 (180 kDa). *Lanes 3 and 4* give immunoblots of serum samples from two patients with paraneoplastic pemphigus recognizing BPAG 1 (*lane 3*), Envoplakin (ENV, 210 kDa) and the 195 kDa periplakin (PPL). *Lane 10* gives the reaction with the monoclonal anti-IQGAP1 antibody at 190 kDa. *Lane 1* shows the reactivity of desmoglein 1 (DG, 160 kDa).

Additional controls with sera of 10 healthy volunteers did not stain any specific band. When lysates of normal human epidermis were immunoblotted with the same sera, we obtained identical results (not shown).

Using a BP180 ELISA kit, we examined the sera of patients 1–5, seven BP sera and two normal human sera. Patient 5 in the ELISA exhibited autoantibodies directed against BPAG 2 with an anti-BP180 value of 97. Sera of patients 1–4 and normal human sera had unit values less than 9, which was according to the manual the threshold value for positivity. The BP control sera had unit values from 10 to 104.

Identification of IQGAP1, a human RasGAP-like protein as antigenic target for autoantibodies To define the protein recognized by circulating autoantibodies NP-40 protein lysates of cultured human keratinocytes were separated electrophoretically. Gel slices containing the protein of interest were localized, excised, and used for protein analysis. Protein identification was based on tryptic digestion and mass spectrum analysis of the generated peptides (performed by Protana, Odense, Denmark). Amino acid sequence analysis of 40 peptide fragments of various length revealed 100% homology to IQGAP1 (**Fig 4**).

To certify further the identity of the antigen we performed immunoprecipitation experiments with a bonified mouse monoclonal anti-IQGAP1 antibody. In a first step, we immunoprecipitated IQGAP1 from NP-40 lysates of cultured human keratinocytes. The precipitated proteins were separated electrophoretically and transferred on to nitrocellulose. When strips of nitrocellulose were immunoblotted with serum samples from the five patients, the autoantibodies bound to a single polypeptide at 190 kDa (**Fig 5*a***, lanes 1–5). Immunoblotting of the precipitated protein with the monoclonal anti-IQGAP1 antibody (**Fig 5*a***, lane 7) and additionally, a polyclonal anti-IQGAP1 antibody showed reactivity (**Fig 5*a***, lane 6) with the same protein, but serum samples of further BP patients (not shown), several control sera of healthy volunteers (**Fig 5*a***, lanes 9–10), and paraneoplastic pemphigus (**Fig 5*a***, lane 8) did not bind to precipitated IQGAP1. As additional control, this experiment was performed using the monoclonal antibody and NP-40 lysis buffer alone for precipitation, and subsequent immunoblotting giving no specific results (**Fig 5*a***, lanes 11–15).

In a next step, immunoprecipitated proteins of C-14-labeled human keratinocytes using patients' sera, the monoclonal anti-IQGAP1 antibody and controls were transferred on to a nitrocellulose membrane. Thereafter, the whole membrane was immunoblotted using mouse monoclonal anti-IQGAP1 antibody, giving a distinct reactivity at 190 kDa (**Fig 5*b***). Afterwards, the nitrocellulose membrane was exposed for autoradiography. Sera of patients 1–4 precipitated only the antigen at 190 kDa, serum of patient 5 also reacted with BPAG 1 (not shown).

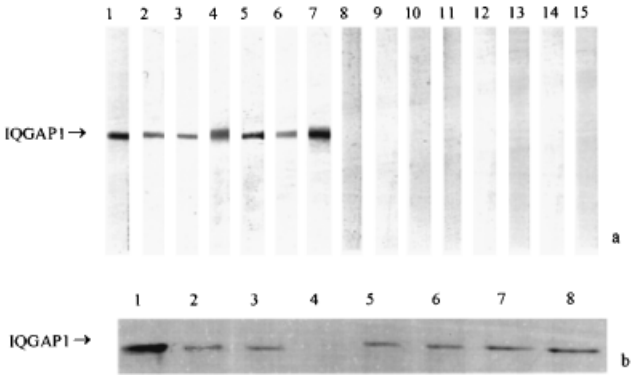

Figure 5. Immunoprecipitation studies of IQGAP1 antibodies. (*a*) When IQGAP1 was precipitated with monoclonal anti-IQGAP1 antibody from lysates of cultured keratinocytes, transferred and immunoblotted with the serum samples of patients 1 to 5 (*lanes 1–5*), with the rabbit anti-IQGAP1 antibody (*lane 6*), and with the mouse monoclonal anti-IQGAP1 antibody (*lane 7*), specific reactivity for IQGAP1 was observed in all cases. For control, immunoblotting with serum of paraneoplastic pemphigus patient was negative (*lane 8*). *Lanes 9 and 10* show immunoblotting with normal human control sera, *lanes 11–15* show immunoblotting with patients' sera when immunoprecipitation was performed with murine anti-IQGAP1 antibody and lysis buffer alone. (*b*) For the reverse experiment, cultured human keratinocytes were precipitated with the patients' sera (*lanes 3 and 5–8*), the monoclonal anti-IQGAP1 antibody (*lane 1*), the polyclonal anti-IQGAP1 antibody (*lane 2*), and a normal human control serum (*lane 4*). After gel electrophoresis and transfer on to nitrocellulose, the membrane was immunoblotted with the monoclonal anti-IQGAP1 antibody. A distinct single band could be observed at 190 kDa within all lanes except the control serum.

MSAADEVDGLGVARPHYGSVLDNERLTAEEMDERRRQNVAYEYLCHLEEA
KRWMEACLGEDLPPTTELEEGLRNGVYLAKLGNFFSPKVVSLKKIYDREQ
TRYKATGLHFRHTDNVIQWLNAMDEIGLPKIFYPETTDIYDRKNMPRCIY
CIHALSLYLFKLGLAPQIQDLYGKVDFTEEEINNMKTELEKYGIQMPAFS
KIGGILANELSVDEAALHAAVIAINEAIDRRIPADTFAALKNPNAMLVNL
EEPLASTYQDILYQAKQDKMTNAKNRTENSERERDVYEELLTQAEIQGNI
NKVNTFSALANIDLALEQGDALALFRALQSPALGLRGLQQQNSDWYLKQL
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LMKLKAQAHAENNEFITWNDIQACVDHVNLVVQEEHERILAIGLINEALD
EGDAQKTLQALQIPAAKLEGVLAEVAQHYQDTLIRAKREKAQEIQDESAV
LWLDEIQGGIWQSNKDTQEAQKFALGIFAINEAVESGDVGKTLSALRSPD
VGLYGVIPECGETYHSDLAEAKKKKLAVGDNNSKWVKHWVKGGYYYYHNL
ETQEGGWDEPPNFVQNSMQLSREEIQSSISGVTAAYNREQLWLANEGLIT
RLQARCRGYLVRQEFRSRMNFLKKQIPAITCIQSQWRGYKQKKAYQDRLA
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INKQKGGLKALSKEKREKLEAYQHLFYLLQTNPTYLAKLIFQMPQNKSTK
FMDSVIFTLYNYASNQREEYLLLRLFKTALQEEIKSKVDQIQEIVTGNPT
VIKMVVSFNRGARGQNALRQILAPVVKEIMDDKSLNIKTDPVDIYKSWVN
QMESQTGEASKLPYDVTPEQALAHEEVKTRLDSSIRNMRAVTDKFLSAIV
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MKGKKSKKISLKYTAARLHEKGVLLEIEDLQVNQFKNVIFEISPTEEVGD
FEVKAKFMGVQMETFMLHYQDLLQLQYEGVAVMKLFDRAKVNVNLLIFLL
NKKFYGK

Figure 4. IQGAP1 is identified as target antigen for autoantibodies. Peptide fragments matching homology with IQGAP1 (SWISS-PROT database) are underlined and represented by the amino acids in bold letters.

Immunofluorescence analysis of IQGAP1 antibodies on normal human skin Using the serum autoantibodies and the anti-IQGAP1 antibody for indirect immunofluorescence experiments on normal human skin, we obtained a combination of a diffuse cytoplasmic pattern, more concentrated in the upper epidermal layers, and a dotted staining at the periphery of individual keratinocytes throughout all cell layers. We observed this staining pattern with serum samples of patients 1–4 (**Fig 6*a***). A similar pattern was found with the monoclonal anti-IQGAP1 antibody (**Fig 6*c***). Serum 5 showed additional weak reactivity along the basement membrane zone of normal human skin (**Fig 6*b***).

IQGAP1 expression in cultured human keratinocytes Double labeling immunofluorescence studies were performed with serum samples and the monoclonal anti-IQGAP1 antibody. Cultured human keratinocytes were grown

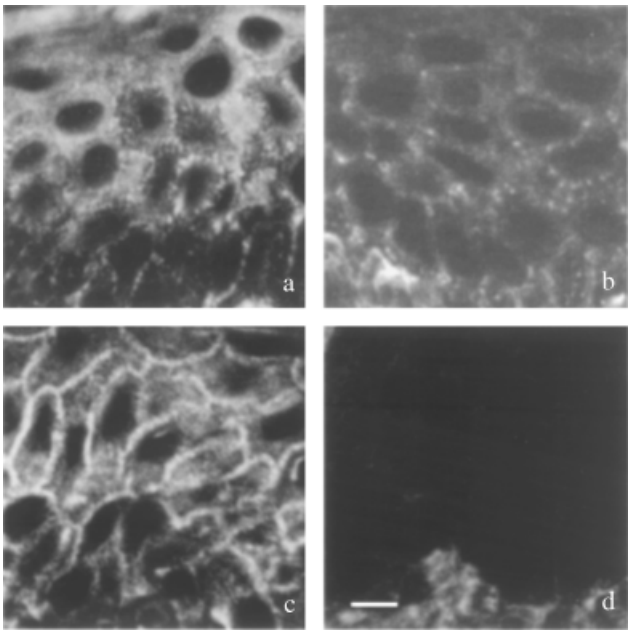

Figure 6. Indirect immunofluorescence on normal human skin. (*a*) Indirect immunofluorescence of patient 1 shows a cytoplasmic staining pattern of the epidermal keratinocytes. At the cell periphery focally dotted staining can be observed throughout the epidermis. Similar reactivity is seen with the serum of patient 5, whereas some reactivity is also seen along the basement membrane zone (*b*). (*c*) For comparison, the monoclonal anti-IQGAP1 antibody is used for immunofluorescence on normal human skin. There is a cytoplasmic staining and labeling of the cell periphery. Normal human serum shows no specific reactivity. *Scale bar*: 20 μm.

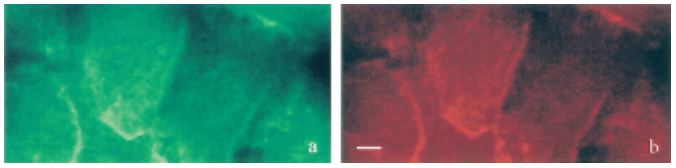

Figure 7. Double immunofluorescence on cultured human keratinocytes. (*a*) Serum of patient 1 and (*b*) the monoclonal anti-IQGAP1 antibody give staining within individual keratinocytes in a cytoplasmic pattern and at the cell periphery at adhesive sites of neighboring keratinocytes. *Scale bar*: 20 μm.

to confluence and permeabilized with Triton-X 100. Immunofluorescence with the monoclonal antibody demonstrates IQGAP1 in cultured cells in a perinuclear localization, and, also, at cell–cell adhesive sites in the periphery of the cells. We found concordance of serum sample staining behavior and the monoclonal anti-IQGAP1 antibody. Cells displayed a cytoplasmic staining pattern, and, in part, a distinct fluorescence signal concentrated at adhesive sites of neighboring keratinocytes (**Fig 7*a*,*b***).

DISCUSSION

In this study, we show the expression of IQGAP1, a newly described RasGAP-like protein (Weissbach *et al*, 1994) in human keratinocytes, and, demonstrate the presence of circulating autoantibodies directed against IQGAP1 in patients with a bullous skin disorder. Direct immunofluorescence of perilesional skin showed bound IgG in a dot-like pattern along the periphery of individual keratinocytes indicative for IgG deposition at adhesive sites of neighboring keratinocytes, and additionally C3 along the basement membrane zone. Indirect immunofluorescence on monkey esophagus gave a cytoplasmic pattern along the periphery of basal cells. These immunomorphologic findings suggested the presence of circulating autoantibodies directed against epidermal antigens in our patients. Immunoblot analysis on lysates of human keratinocytes revealed antibody binding to a 190 kDa antigen in all five patients. Amino acid sequence analysis and further immunoprecipitation studies using a bonified monoclonal antibody identified the protein as IQGAP1 (Weissbach *et al*, 1994), a protein suspected to act as an effector molecule for Cdc 42 and Rac 1, members of the small GTPase family, and also thought to play a part in E-cadherin-mediated cell adhesion (Kuroda *et al*, 1998; Kaibuchi *et al*, 1999). The complex of antigens characteristic for paraneoplastic pemphigus also includes a protein at approximately 190 kDa, periplakin. This is of further interest as sera of some nonparaneoplastic pemphigus patients, e.g., BP, have been shown to react with periplakin (Nagata *et al*, 2001). Using a 5% SDS–PAGE for immunoblotting and immunoprecipitation as running condition, however, there is a striking difference between periplakin (Ma and Sun, 1986) of control paraneoplastic pemphigus sera and IQGAP1. Additionally, two-dimensional gel electrophoresis followed by immunoblotting with sera of patients 1–4 and the monoclonal antibody showed identical reactivity at about 190 kDa and pI between 6 and 7 (not shown). IQGAP1 is a large protein with IQ domains, sequence similarity to RasGAP-like proteins (Weissbach *et al*, 1994) and several protein binding motifs for direct binding to calmodulin (Ho *et al*, 1999; Li *et al*, 1999; Briggs *et al*, 2002), actin (Erickson *et al*, 1997), the small GTPases Cdc42 and Rac1 (Kuroda *et al*, 1996; Ho *et al*, 1999), E-cadherin (Kuroda *et al*, 1998), and β-catenin (Kuroda *et al*, 1998; Briggs *et al*, 2002) thus providing evidence for fundamental cellular metabolic and modulating effects as well as playing a key part in regulating E-cadherin-mediated cell–cell adhesion and cytoskeletal organization (Kuroda *et al*, 1998; Kaibuchi *et al*, 1999). IQGAP1 has been reported to interact with the N-terminal region of β-catenin, which contains the α-catenin binding domain and to compete with α-catenin for binding to β-catenin and, if not bound to activated Cdc42 or Rac1, IQGAP1 is able to dissociate α-catenin from the catenin–cadherin complex negatively regulating E-cadherin-mediated cell–cell adhesion (Kuroda *et al*, 1998; Kaibuchi *et al*, 1999). Depending on its binding mode to linkage proteins, IQGAP1 can promote or inhibit cadherin-based cell adhesion (Kaibuchi *et al*, 1999; Briggs *et al*, 2002). By double immunofluorescence on cultured keratinocytes with serum antibodies and the monoclonal anti-IQGAP1 antibody we found subcellular colocalization of the respective antibodies. The expression of IQGAP1 in human keratinocytes reported here was found at cell–cell adhesive sites in the periphery of the cell close to the plasmalemma and in a perinuclear distribution within the cytoplasma, a pattern described for IQGAP1 in various other cells (Kuroda *et al*, 1996, 1998; Erickson *et al*, 1997; Li *et al*, 1999) where it is found associated with Golgi membranes and the cytoskeleton near plasmalemma anchored adhesion molecules (Kuroda *et al*, 1996; Ho *et al*, 1999). According to these observations, in human keratinocytes IQGAP1 may contribute to the organization and integrity of the epidermal layer.

We here report of IQGAP1 in human keratinocytes, and of autoantibodies directed to this antigen in association with a bullous skin disorder. By immunoblotting and immunoprecipitation lysates of keratinocytes with the 205 serum samples we did not find a 190 kDa antigen recognized by any sera of pemphigus vulgaris or pemphigus foliaceus, but only by sera of our five presented patients diagnosed as erythema multiforme (or erythema multiforme-like eruption) and BP. Clinically, the patients presented plaques, smaller and larger blisters, reminiscent of erythema multiforme and BP. Histology revealed subepidermal blistering. Lesions showed linear C3 along the basement membrane zone. Only one of the five patients described had additionally autoantibodies against a 230 kDa protein, supposed to be BPAG 1, and also showed linear IgG along the basement membrane zone in direct and indirect immunofluorescence. As demonstrated by BP180 ELISA, the serum from that patient

reacted with BPAG 2, too. Determination of subclasses revealed IgG1 (and IgG4) autoantibodies for potential complement binding activity (not shown). There are, however, some discrepancies in clinical, biochemical, and immunomorphologic presentation of the five patients, which show features of erythema multiforme and BP. It is possible that further anti-BP 180 antibodies are beyond detection for biochemical methods, but could induce C3 deposition and induce pathogenic processes. These findings strongly suggest that anti-IQGAP1 autoantibody-associated disease is related to or appears together with BP.

Autoantibodies in the pemphigus or pemphigoid disease are directed in part against extracellular portions of the transmembranous target proteins that makes it easy to accept that an antibody–antigen reaction could initiate the pathogenic events (Hertl *et al*, 1998; Hertl, 2000; Lin *et al*, 2000). The autoantibodies presented here are directed against an intracellularly located antigen, a topographically much more controversial site to explain the possible pathogenic relevance (Alarcón-Segovia *et al*, 1996). Autoimmune diseases, such as lupus erythematosus, however, which can be viewed as a prototype for the breakdown of tolerance to a wide spectrum of autoantigens are characterized by autoantibodies directed against intracellular localized target molecules (Alarcón-Segovia *et al*, 1996; Sontheimer, 1999; Kamradt and Mitchison, 2001). Studies with anti-nuclear antibodies of patients with systemic lupus erythematosus indicate IgG antibody penetration into living epithelial cells via receptor-mediated endocytosis as a possible entrance mechanism (Golan *et al*, 1993). Reports on pathogenic antibodies binding to glucose-6-phosphatase isomerase, a glycolytic enzyme localized intracellularly that induce autoimmune arthritis (Matsumoto *et al*, 1999) indicate the existence of a different mechanism of autoimmunity. A recent report on *in vivo* function of IQGAP1 showed that mice lacking IQGAP1 develop gastric hyperplasia (Lin *et al*, 2000). Further studies showed that localization of IQGAP1 is inversely correlated with E-cadherin mediated intercellular adhesion in gastric cancers (Takemoto *et al*, 2001), IQGAP1 overexpression in human colorectal carcinomas (Nabeshima *et al*, 2002), and that IQGAP1 is upregulated in gastric cancer cells (Kamiyama and Inazawa, 2001) implying that IQGAP1 may play a part in cancer development by modulating tumor cell adhesion (Briggs *et al*, 2002). Considering these reports and the history of patients 1 (gastritis), 2 (colitis ulcerosa), and 4 (colon carcinoma 7 y before) the intriguing question arises whether the development of anti-IQGAP1 autoantibodies is of any importance in this context. It might be possible that in the course of the gastroenteral disease exposure of this intracellular antigen could lead to subsequent antibody development. Considering the complex *in vivo* function of IQGAP1, one could imagine that autoantibodies once inside the cell might also interfere with binding motifs critically disturbing cell adhesive mechanisms (Fukata *et al*, 2001). In that case, a local inflammatory reaction of the skin could support autoantibodies directed against intracytoplasmic antigens to enter cells. Therefore, it could be that IQGAP1 autoantibodies at the same time represent an "epiphenomenon", which during the course of another autoimmune bullous skin disease such as BP, circumstantially gain functional relevance in perturbing a pathogenic process. Addressing these questions will lead to further investigations involving experimental serum transfer studies and development of an animal model of disease.

In summary, we demonstrate that IQGAP1, a newly described multifunctional protein involved in cellular organization and cell adhesion, is constitutively expressed by human keratinocytes. Furthermore, we found autoantibodies in sera of patients with distinct blistering skin eruptions potentially targeting that Ras GTP-ase-like protein.

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