Goodpasture Antigen-binding Protein, the Kinase That Phosphorylates the Goodpasture Antigen, Is an Alternatively Spliced Variant Implicated in Autoimmune Pathogenesis*

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The non-collagenous C-terminal domain of the α3 chain of collagen IV is the autoantigen in Goodpasture disease, an autoimmune disorder described only in humans. Specific N-terminal phosphorylation is a biological feature unique to the human domain when compared with other homologous domains lacking immunopathogenic potential. We have recently cloned from a HeLa-derived cDNA library a novel serine/threonine kinase (Goodpasture antigen-binding protein (GPBP)) that phosphorylates the N-terminal region of the human domain (Raya, A. Revert, F. Navarro, S. and Saus J. (1999) J. Biol. Chem. 274, 12642–12649). We show here that the pre-mRNA of GPBP is alternatively spliced in human tissues and that the most common transcript found encodes GPBP-D26, a molecular isoform devoid of a 26-residue serine-rich motif. Recombinantly expressed GPBP-D26 exhibits lower activity than GPBP, due at least in part to a reduced ability of GPBP-D26 to interact and to form very active high molecular weight aggregates. In human tissues, GPBP shows a more limited expression than GPBP-D26 but displays a remarkable preference for the small vessels and for histological structures targeted by natural autoimmune responses including alveolar and glomerular basement membranes, the two main targets in Goodpasture disease. GPBP expression is, in turn, up-regulated in the striated muscle of a Goodpasture patient and in other autoimmune conditions including cutaneous lupus erythematosus, pemphigoid, and lichen planus.

Goodpasture (GP)1 disease is an exclusive human disorder

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¹ The abbreviations used are: GP, Goodpasture; bp, base pair; CLE, cutaneous lupus erythematosus; GPBP and GPBP-D26, Goodpasture antigen-binding protein and its alternative splicing variant devoid of the exon-encoded 26-residue motif; rGPBP and rGPBP-D26, recombinant counterparts of GPBP and GPBP-D26; HPLC, high performance liquid chromatography; kb, thousand base pairs; NC1, non-collagenous C-terminal domain; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase-coupled polymerase chain reaction; SLE, systemic lupus erythematosus; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Peptides—GPpep1, KKKGKDSGSPATWTTRGFVFT, representing residues 3–23 of the human α3(IV)NC1 was synthesized by Chiron. GPpep1, PYRSRSMSSSILVSASSDDVHRFSSQ, representing residues 271–396 of GPBP was synthesized by Genosys. FLAG peptide, DYKDDDDK, was from Sigma.

Oligonucleotides—The following oligonucleotides were synthesized from Life Technologies, Inc.: ON-GPBP-11m, G CGG GAC TCA GCC GCC GGA TTT TTCT; ON-GPBP-15m, AC AGC TGG CAG AGA GA; ON-GPBP-20c, C ATG GGT AGC TTT TAA AG; ON-GPBP-22m, TA GAA GAG TCA CAG AGT GAA AAG G; ON-GPBP-53c, GAA TCT GAA CAA AAT AGG CTT CT; ON-GPBP-56m, CCC TAT AGC CGC TCT TC; ON-GPBP-57c, CTG GGA GCT GAA TCT GT; ON-GPBP-62c, GTG GTT CTG CAT CTC TCT AAC; ON-GPBP-D26, CA CAT AGA TTT GTG CAA AAG GTT GAA GAG ATG GTG CAG AAC.

Plasmid Construction, Expression, and Purification of Recombinant Proteins—The plasmid pHiL-FLAG-n4, used for recombinant expression of GPBP-charged FIIa, has been described elsewhere (4). The sequencing code for the 78-bp exon was deleted by site-directed mutagenesis using ON-GPBP-D26 to generate the plasmid...
precipitated by adding 20% (w/v) of sodium sulfate and centrifugation (25 min at 10,000 g) at 4 °C, the solution was clarified by centrifugation (Fig. 1 B; lanes 1 and 3). Numbers on the left and right refer to molecular weight in base pairs. B, the region missing in the muscle transcript was identified, and its nucleotide cDNA sequence (lowercase) and deduced amino acid sequence (upercase) were determined. The genomic DNA comprising the cDNA region of interest was sequenced, and its structure is drawn in C.

The location and relative sizes of the 78-bp exon spliced out in GPBPΔ26 (black box), adjacent exons (gray boxes), and introns (lines) are represented. The size of both intron and exons is given, and the nucleotide sequence of intron-exon boundaries is presented with the consensus for 5' and 3' splice sites shown in boldface.

PhIL-FLAG-m4Δ26. Expression and affinity purification of the recombinant proteins, rGPBP and rGPBPΔ26 and human α3(IV)NC1, was done as in Ref. 4.

HPLC Gel Filtration—Samples of 250 μl were injected into a gel filtration PE-TSK-G4000SW HPLC column equilibrated with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. The material was eluted from the column at 0.5 ml/min and monitored at 220 nm, and fractions were collected every minute.

In Vitro Phosphorylation Assays—The autophosphorylation, transphosphorylation, and in-blot renaturation studies were performed as in Ref. 4.

Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared as described previously (5) and retrotranscribed (5 μg) using Ready-To-Go You-Prime First-Strand beads (Amer sham Pharmacia Biotech) and 40 pmol of ON-GPBP-53c. The corresponding cDNA was subjected to PCR using primers ON-GPBP-11 m/ON-GPBP-53c or ON-GPBP-15 m/ON-GPBP-62c. The products obtained with 15 m-62c and containing the 78-bp exon were further identified by Alul digestion. To amplify specifically GPBP transcripts, PCR was performed using primers ON-GPBP-15 m/ON-GPBP-57c.

Northern Hybridization Studies—Pre-made Northern blots (CLONTECH) representing multiple human tissues or human-derived tumor cell lines were probed with a cDNA containing the 78-bp exon present only in GPBP or with a 261-bp cDNA fragment representing the flanking regions (104-bp 5' and 157-bp 3') of the 78-bp exon common to both isoforms. The corresponding cDNA fragments were obtained by PCR using the pair of primers ON-GPBP-56m and ON-GPBP-57c using GPBP as a template or with primers ON-GPBP-22m and ON-GPBP-20c using GPBPΔ26 as a template. The resulting products were random-labeled and hybridized following the manufacturer's instructions.

Antibodies—Polyclonal antibodies were raised in chicken against a synthetic peptide (GPBP pep1) representing the sequence coded by the 78-bp exon (Genosys). Egg yolks were diluted 1:10 in water, and the pH was adjusted to 5.0. After 6 h at 4 °C, the solution was clarified by centrifugation (25 min at 10,000 g at 4 °C), and the antibodies were precipitated by adding 20% (w/v) of sodium sulfate and centrifugation at 20,000 × g for 20 min. The pellets were dissolved in phosphate-buffered saline (1 ml per yolk) and used for immunohistochemical studies. The production of antibodies against GPBP/GPBPΔ26 has been previously reported (4).

Immunohistochemical Techniques—SDS-PAGE and Western blotting were performed under reducing conditions as in Ref. 3. For Western purposes, human recombinant α3(IV)NC1 (4) was analyzed by SDS-PAGE under reducing conditions, transferred to Immobilon P (Millipore), renatured in Tris-buffered saline in the presence of Tween 20 (0.05%), and probed for 1 h at 37 °C with 30 μg/ml of either rGPBP or rGPBPΔ26 in the same buffer. Bound material was detected using GPBP/GPBPΔ26-specific monoclonal antibodies (monoclonal antibody 14) and an immunoperoxidase-based conjugate.

Immunohistochemical studies were done on formalin-fixed paraffin-embedded or frozen human biopsies fixed with cold acetone using an immunoperoxidase-based method (6) or standard procedures for indirect immunofluorescence. Skin biopsies from 6 healthy, 11 non-autoimmune dermatitis or vasculitis, 2 lichen planus, 5 CLE, 1 pemphigus, and 1 pemphigoid were independently analyzed using one of the chemical or immunochromical approaches indicated. In addition to clinical diagnosis, all the patients had histological diagnosis confirmation except for the pemphigus.

Sedimentation Velocity and Equilibrium—Determination of sedimentation velocities were performed in an Optima XL-A analytical ultracentrifuge (Beckman Instruments Inc.), equipped with a VIS-US scanner, using a Ti60 rotor and double sector cells of Epon-charcoal of 12-mm optical path length. Samples of ~400 μl were centrifuged at 30,000 rpm and 20 °C, and radial scans at 220 nm were taken every 5 min. The sedimentation coefficients were obtained from the rate of movement of the solute boundary using the program XLAVEL (supplied by Beckman).

Sedimentation equilibrium experiments were done as described above for velocity experiments using samples of 70 μl and centrifugation at 8,000 rpm. The experimental concentration gradients at equilibrium were analyzed using the program EQASSOC (Beckman) to determine the corresponding weight average molecular mass. A partial specific volume of 0.711 cm³/g for GPBP and 0.729 cm³/g for GPBPΔ26 were calculated from the corresponding amino acid compositions. These studies were performed at the Analytical Ultracentrifugation facilities of the Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas, Madrid, Spain).

Two-hybrid Studies—Self-interaction studies were carried out in Saccharomyces cerevisiae (HF7c) using pGBT9 and pGAD424 plasmids (CLONTECH) to generate GAL4-binding and -activation domains fusion proteins, respectively. Interactions were assessed following the manufacturer’s recommendations. The β-galactosidase activity was assayed with 0.75 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in N,N-dimethylformamide, for the filter assays and with ortho-nitrophenyl-β-D-galactopyranoside (0.64 mg/ml) for the liquid determinations.

RESULTS

Identification of Two Spliced GPBP Variants—To characterize GPBP in human tissues, we performed RT-PCR on total RNA using specific oligonucleotides that flank the full open reading frame of GPBP. A single cDNA fragment displaying a lower size than expected was obtained from skeletal muscle (Fig. 1A), and also from kidney, lung, skin, and adrenal gland (not shown). We fully sequenced the 2.2-kb cDNA from human muscle (GenBank™ accession number AF232930), and we
found it identical to HeLa-derived material except for the absence of a 78-bp fragment (positions 1519–1596) that encodes a 26-residue motif (371–396 amino acids) (Fig. 1B). We therefore named this more common isoform of GPBP as GPBP\(\Delta 26\). By combining nested PCR re-amplifications and endonuclease restriction mapping, we determined that the RT-PCR products obtained in every human tissue tested corresponded to the same molecular species (not shown).

To investigate whether the 78-bp fragment represents an exon skipped during pre-mRNA processing, we used this cDNA fragment to probe a human-derived genomic library, and we isolated an 14-kb clone. By combining Southern blot hybridization and PCR, the genomic clone was characterized, and a contiguous DNA fragment of 12,482-bp was fully sequenced (GenBank\(^{\text{TM}}\) accession number AF232935). The sequence contained from 5’ to 3’, 767 bp of intron sequence, a 93-bp exon, an 818-bp intron, the 78-bp exon of interest, a 9650-bp intron, a 818-bp intron, the 78-bp exon of interest, a 9650-bp intron, a 980 bp of intron sequence (Fig. 1C). The exon/intron boundaries determined by comparing the corresponding DNA and cDNA sequences meet the canonical consensus for 5’ and 3’ splice sites (Fig. 1C) (7), thus confirming the exon nature of the 78-bp sequence.

We assessed the relative expression of GPBP and GPBP\(\Delta 26\) by Northern blot analysis (Fig. 2). Both isoforms were preferentially expressed in striated muscle (skeletal and heart) and brain and poorly expressed in placenta, lung, and liver. However, in kidney and pancreas as well as in the cancer cell lines, the molecular species containing the 78-bp exon (GPBP) were relatively expressed at much lower levels than those devoid of the exon (GPBP\(\Delta 26\)). Note that in Fig. 2 we used more permissive conditions to enhance hybridization of the GPBP-specific probe since no signal was detectable when washing at stringent conditions (not shown).

All the above indicate that GPBP is expressed at low levels in normal human tissues, and therefore the initial failure to detect GPBP by RT-PCR in different human tissues can be attributed to a preferential amplification of the more abundant GPBP\(\Delta 26\). Accordingly, the cDNA of GPBP was obtained from human tissues (skeletal muscle, lung, kidney, skin, and adrenal gland) when 78-bp exon-specific primers were used in the PCR (not shown). Finally, quantitative RT-PCR studies on total RNA from human skeletal muscle reveal that the molecular species containing the 78-bp exon (GPBP) represent less than 10% of the molecular species devoid of it (GPBP\(\Delta 26\)) (not shown). Thus in the Northern blot studies shown in Fig. 2, the major transcript detected when using the cDNA probe that represents both isoforms was GPBP\(\Delta 26\).

**Recombinant Expression and Functional Characterization of GPBP\(\Delta 26\)**—To investigate whether the absence of the 26-residue serine-rich motif would affect the biochemical properties of GPBP, we expressed and purified both isoforms (rGPBP and rGPBP\(\Delta 26\)), and we assessed auto- and trans-phosphorylation activities along with their ability to bind recombinant \(\alpha3(IV)\text{NC1}\) (Fig. 3). SDS-PAGE and Western blot analysis revealed that purified materials contained a major single polypeptide and several related products, which number, relative amounts, and molecular weight in rGPBP\(\Delta 26\) varied with respect to rGPBP (Fig. 3A). However, these differences including the molecular weight displayed by the major polypeptide product (89 kDa for GPBP and 84 kDa for GPBP\(\Delta 26\)) could not only be attributed to the mere presence or absence of the 26-residue, thus revealing the existence of important structural differences between GPBP and GPBP\(\Delta 26\). These structural differences were responsible at least in part for the higher in-solution activity displayed by rGPBP, which was more efficient in both autophosphorylation and phosphorylating the N-terminal region of the human \(\alpha3(IV)\text{NC1}\) domain (Fig. 3B). The phosphate transfer activities of rGPBP and rGPBP\(\Delta 26\) were, however, very similar after SDS-PAGE and *in situ* renaturation studies (Fig. 3C), indicating that the structural features that depend on the presence or absence of the 26-residue motif could not be efficiently renatured. The higher activity of GPBP was also confirmed by showing higher binding of this isoform to recombinant human \(\alpha3(IV)\text{NC1}\) domain in specific far Western studies (Fig. 3D). Renaturation studies further show that kinase activity resides at the major polypeptides representing the proposed open reading frames and is not detectable at the derived products.

**rGPBP and rGPBP\(\Delta 26\) Exist as Very Active High Molecular Weight Aggregates**—Gel filtration analysis of rGPBP or rGPBP\(\Delta 26\) yielded two chromatographic peaks I and II (Fig. 4), both displaying higher molecular weight than expected for the major individual molecular species as determined on SDS-PAGE studies (89 and 84 kDa, respectively). The bulk of the

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2 Similar pattern to that shown in Fig. 3D was obtained when assessing the binding of rGPBP or rGPBP\(\Delta 26\) to recombinant proteins representing the human highly homologous \(\alpha1\) and \(\alpha6(IV)\text{NC1}\) domains; however, no significant material bound to the recombinant material representing the \(\alpha2\), \(\alpha4\), and \(\alpha6(IV)\text{NC1}\) counterparts.
Fifteen microliters of the indicated fractions were subjected to SDS-PAGE and Coomassie Blue-stained or incubated in the presence of \(^{32}\)P-ATP and analyzed by immunoblot and autoradiography. The material in every chromatographic peak contained the primary polypeptide and minor related products (not shown in Fig. 4 composite), indicating that the primary polypeptide associates to form high molecular weight aggregates mainly stabilized by non-covalent bonds. The kinase activity also exhibited two peaks coinciding with the chromatographic profiles; however, the material eluted in peak I showed a much higher specific activity than the material present in peak II, indicating that these high molecular weight aggregates contained a much more active form of the kinase. In the study shown, equal volumes of rGPBP fractions number 13 and 20 exhibited comparable phosphorylating activity, despite that the protein content was ~20 times lower in fraction 13 as estimated by Western blot and Coomassie Blue staining (Fig. 4A). The specific activities of rGPBP and rGPBPΔ26 at peak II are also different and consistent with the studies shown for the whole material, thus supporting that the presence of the 26-residue serine-rich motif renders a more active kinase. These results also suggest that both rGPBP and rGPBPΔ26 exist as oligomers under native conditions and that both high molecular weight aggregation and specific activity are greatly dependent on the presence of the 26-residue serine-rich motif. Analytical centrifugation analysis of rGPBP revealed that peak I contained large aggregates (over 10^7 Da). Peak II of rGPBP contained a homogeneous population of 220 ± 10 kDa aggregates likely representing trimers with a sedimentation coefficient of 11 S. Peak II of rGPBPΔ26, however, consisted of a more heterogeneous population that likely contains several oligomeric species. The main population (~80%) displayed a weight average molecular mass of 310 ± 10 kDa and a coefficient of sedimentation of 14 S.

**GPBP and rGPBPΔ26 Self-interact in a Yeast Two-hybrid System**—To assess the physiological relevance of the self-aggregation and to dig into the role of the 26-residue motif, we performed comparative studies using a two-hybrid interaction system in yeast. In this type of studies the polypeptides whose interaction is under study are expressed as a part of a fusion protein containing either the activation or the binding domains of the transcriptional factor GAL4. An effective interaction between the polypeptides under study would result in the reconstituent of the transcriptional activator and the subsequent expression of the two reporter genes \(\text{lacZ}\) and \(\text{his3}\) genes, allowing colony color detection and growth in a His-defective medium, respectively. We estimated the intensity of interactions by the growth rate in histidine-defective medium in the presence of different concentrations of a competitive inhibitor of the \(\text{his3}\) gene product (3-aminotriazole) and a quantitative colorimetric liquid \(\beta\)-galactosidase assay. A representative experiment is presented in Fig. 5. When assaying GPBPΔ26 for self-interaction, a significant induction of the reporter genes

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**Fig. 3.** GPBPΔ26 displays lower phosphorylating and binding activity than GPBP. A, rGPBP (lane 1) or rGPBPΔ26 (lane 2) was subjected to SDS-PAGE under reducing conditions and either Coomassie Blue-stained (2 µg) or blotted (200 ng) with FLAG (α-FLAG) or GPBP/GPBPΔ26 (α-GPBP)-specific antibodies. B, 200 ng of rGPBP (lane 1) or rGPBPΔ26 (lane 2) were in vitro phosphorylated without substrate to assay auto-phosphorylation (left) or with 5 nmol of GPpep1 to determine trans-phosphorylation activity (right). The phosphorylation mixtures were analyzed by SDS-PAGE and autoradiography. An arrowhead indicates the position of the GPpep1. C, 3 µg of GPBP (lane 1) or GPBPΔ26 (lane 2) were bound to human recombinant o3IVNC1 domain (200 ng) as described under “Material and Methods.” The numbers and bars indicate the molecular mass in kDa and the relative position of the molecular weight markers, respectively.

**Fig. 4.** rGPBP and rGPBPΔ26 form very active high molecular weight aggregates. About 300 µg of GPBP (A) or rGPBPΔ26 (B) were subjected to HPLC gel filtration. Arrowheads and numbers, respectively, indicate the elution profile and molecular mass of molecular weight standards used. Larger aggregates eluted in the void volume (I), and the bulk of the material eluted in the separation range of the column as a second peak between 669 and 158 kDa standards (II). Fifteen microliters of the indicated fractions were subjected to SDSPAGE and Coomassie Blue-stained (Coomassie). Five microliters of the same fractions were in vitro phosphorylated, and the reaction was stopped by boiling in SDS sample buffer, analyzed by SDS-PAGE, transferred, and autoradiographed for 1 or 2 h (Kinase assay), and then blotted using anti-FLAG antibodies (Western).
was observed, whereas no expression was detectable when each fusion protein was individually expressed (not shown) or expressed with the partner empty plasmid (Fig. 5). The insertion of the 26-residue motif in the polypeptide resulted in a notable increase in polypeptide interaction allowing significant growth in plates containing 1 mM 3-aminotriazole (Fig. 5A). We arrived to similar conclusions when the interactions for the different plasmid combinations were measured in a β-galactosidase liquid assay (Fig. 5B). These results indicate that GPBP-D26 self-associates in vivo and that the insertion of the 26 residues into the polypeptide chain yields a more interactive molecular species.

**GPBP Is Highly Expressed in Human but Not in Bovine or in Murine Glomerulus and Alveolus—**

By using antibodies that recognize both GPBP and GPBP-D26, we have shown preferential expression of these proteins in human cells and tissues targeted by common autoimmune responses (4). To investigate specifically the expression of GPBP, we raised polyclonal antibodies against a synthetic peptide representing the 26-residue motif characteristic of this isoform (GPBPpep1), and we used them for immunohistochemical studies on formalin-fixed paraffin-embedded human tissues. In some tissues, these antibodies showed more specificity for the autoimmune targets (e.g., the biliary ducts and the Langerhans islets) than the antibodies recognizing both isoforms (not shown). Nevertheless, the most remarkable finding was the presence of linear deposits of antibodies around the small vessels (Fig. 6, A and B), suggesting that GPBP associates with endothelial basement membranes. Consequently, the anti-GPBPpep1 antibodies displayed a strong association with glomerular (Fig. 6, C and D) and alveolar (not shown) basement membranes and were deposited in human glomerulus closely resembling GP autoantibodies (Fig. 6, E and F). These findings further support the initial observation that GPBP is expressed in tissue structures targeted by
natural autoimmune responses (4) and suggest that GPBP expression confers vulnerability to autoimmune attack.

To assess further this hypothesis, we investigated the expression of GPBP and GPBPΔ26 in the kidney of mammals that naturally do not undergo GP disease, and we compared their expression in human renal cortex (Fig. 7). GPBP-specific antibodies failed to stain the vessels and the glomerulus of either bovine or murine specimens (compare Fig. 7, A with B and C). However, antibodies specific for GPBP and GPBPΔ26 stained renal cortex in all three species, although with different distributions and intensities (Fig. 7, D–F). In bovine renal cortex, GPBPΔ26 was relatively less expressed than in human but showed similar tissue distribution. In murine samples, however, GPBPΔ26 displayed a tissue distribution closely re-
seembing that of GPBP in human samples. Similar results were obtained when studying lung samples from the three different species (not shown). To rule out that the differences in the antibody detection were due to primary structure divergence rather than to a differential expression, we determined by cDNA sequencing the corresponding primary structure in these two species (GenBank™ accession numbers AF232931 and AF232932). Bovine and mouse GPBP displayed an overall identity with human material of 97.9 and 96.6%, respectively. Furthermore, the mouse 26-residue motif was identical to human, whereas the bovine motif differed only in one residue. Finally, we successfully amplified GPBP cDNA from mouse or bovine kidney total RNA using 78-bp exon-specific oligonucleotides (not shown), indicating that in these species GPBP is also expressed in the kidney but at levels that are not detectable by the immunological techniques.

**GPBP Is Highly Expressed in Several Autoimmune Conditions**—We analyzed several tissues from different GP patients by specific RT-PCR to assess GPBP/GPBPΔ26 mRNA levels. As in control kidneys, the major expressed isoform in GP kidneys was GPBPΔ26 (not shown). However, GPBP was preferentially expressed in the skeletal muscle of one patient, whereas GPBPΔ26 was the only isoform detected in a control human muscle (Fig. 8A). Quantitative RT-PCR studies supported these findings, and thus in the patient muscle the 78-bp exon-containing mRNAs were approximately 10 times more abundant than GPBPΔ26, whereas these molecular species represented less than one-tenth of the GPBPΔ26 mRNAs in control muscle (not shown).

Since we did not have a kidney sample from this particular patient, we could not assess GPBP/GPBPΔ26 expression in the corresponding target organ. For similar reasons, we could not assess GPBP/GPBPΔ26 expression in the muscle of the patients whose kidneys were analyzed. Nevertheless, the increased levels of GPBP in a GP patient suggest that GPBP/GPBPΔ26 expression is altered during GP pathogenesis and that augmented GPBP expression has a pathogenic significance in autoimmunity.

To investigate the expression of GPBP and GPBPΔ26 in autoimmune pathogenesis, we studied cutaneous autoimmune processes and compared with control samples representing normal skin or non-autoimmune dermatitis (Fig. 8). A variable but limited expression of GPBP restricted to the most peripheral strata of the epidermis was observed in control skins (Fig. 8, B and E). However, keratinocytes expanding from basal to cornu- neum strata expressed abundant GPBP in skin affected by either CLE (Fig. 8, C and F), lichen planus (Fig. 8, D and G), or pemphigoid (not shown). GPBP was expressed in bleb structures at the cell surface (Fig. 8, F and G) previously identified as apoptotic bodies in cultured keratinocytes upon UV irradiation (8). In contrast, antibodies recognizing both GPBP/ GPBPΔ26 yielded a major diffuse cytosolic pattern through the whole epidermis in both autoimmune-affected or control samples (not shown).

The differences in GPBP expression found between control and autoimmune-affected epidermis were also evident when the comparative immunohistochemical studies were done using samples representing non-affected or affected skin regions of individual CLE patients (Fig. 9, A–C). Furthermore, the relative content of GPBP in skin extracts representing CLE-affected regions was significantly augmented in comparison with non-affected regions of the same patient or with control samples (normal and non-autoimmune dermatitis) as determined by specific RT-PCR studies (Fig. 9D).

**DISCUSSION**

Alternative pre-mRNA splicing is a fundamental mechanism for differential gene expression that has been reported to regulate the tissue distribution, the intracellular localization, and the activity of different protein kinases (9–12). Closely resembling GPBP, B-Raf exits as different intracellular variants in which the presence of specific exons results in more interactive, efficient, and oncogenic kinases (13).

Although it is evident that rGPBPΔ26 still bears the uncharacterized catalytic domain of this novel kinase, both binding and phosphorylating activities are greatly reduced when compared with rGPBP. Gel filtration and two-hybrid experiments provide some insights into the mechanisms that underlie such a reduced phosphate transfer activity. About 1–2% of rGPBP is organized in very high molecular weight aggregates that display about one-third of the phosphorylating activity of rGPBP, indicating that molecular aggregation renders a more efficient quaternary structure. Consistently rGPBPΔ26, with virtually no peak 1 material, displays a reduced kinase activity. However, aggregation does not seem to be the only mechanism by which the 26-residue motif increases the specific activity since the rGPBPΔ26 material present in peak II also shows a reduced phosphorylating activity when compared with homologous fractions of rGPBP. Far Western and two-hybrid studies suggest that rGPBP-derived aggregates also display higher specific activities because the insertion of the 26-residue motif renders a more interactive and strengthened quaternary structure. Conformational changes induced by the presence of an exon-encoded motif that alters the activation status of the kinase have been proposed for the linker domain of the Src protein (14) and exons 8b and 10 of B-Raf (13). Alternatively, the 26-residue motif may provide the structural requirements for specific phosphorylation (Ser or Tyr) necessary for full activation of the kinase. In any event, the similar activity displayed by each individual isoform in the in-blot renaturation studies indicates the existence of a common catalytic domain and suggests that GPBPΔ26 and GPBP represent different strategies to regulate its activity.

The primary structure of the α3(IV)NC1 lends itself to a complex folding process that yields multiple conformers (conformational isomers). Non-assembled conformers are metastable structures specifically activated by phosphorylation for supramolecular aggregation and likely quaternary structure formation. Consistently, the α3(IV)NC1 domain of GP patients shows conformational alterations that are specifically recognized by the pathogenic autoantibodies, suggesting that altered conformers elicited the autoimmune response. In this scenario, three features of the human system place the conformational process of the α3(IV)NC1 domain in a vulnerable condition that, in turn, predispose humans for GP disease. 1) The N terminus of the human α3(IV)NC1 contains a phosphorylatable motif shared by cAMP-dependent protein kinase and GPBP (2–4). 2) The human α3(IV) gene generates multiple products by alternative exon splicing (5, 15). Exon skipping generates alternative products with divergent C-terminal ends that up-regulate the cAMP-dependent protein kinase phosphorylation of the primary α3(IV)NC1 product. 3) GPBP is expressed and associated with the two major targets of the GP autoantibodies, glomerular and alveolar basement membranes. So far, all the GP kidneys studied expressed

3 A. Raya, F. Revert, C. Tárrega, T. Hellmark, A. Boutaud, J. Cervera, J. R. Penadés, J. Wieslander, B. G. Hudson, S. Quinones, Y. Ninomiya, and J. Saus, manuscript in preparation.

4 J. R. Penadés, A. Raya, and J. Saus, unpublished results.
higher levels of the alternative product (5), and an augmented expression of GPBP has been found in a GP patient. Both conditions are expected to increase the phosphorylation of the α3(IV)NC1 domain and therefore to influence the corresponding conformational process.

Keratinocytes, during maturation from basal to corneum strata, undergo an apoptosis-dependent differentiation process (16, 17). Cell surface bleb formation is a well-established step between nuclear condensation and cytoplasmic contraction in the apoptotic morphological cascade of many cell systems including keratinocytes (8). In the epidermis, GPBP is associated with cell surface blebs of keratinocytes suggesting that GPBP expression and apoptosis are related processes. Keratinocytes from SLE patients show a remarkably increased sensitivity to UV-induced apoptosis (8, 18, 19), and a premature and aberrant apoptosis of the basal keratinocytes has been reported to occur in SLE and dermatomyositis (20). Consistently, we found GPBP expressed in apoptotic bodies expanding from basal to peripheral strata in the epidermis affected by different autoimmune processes including CLE, pemphigus, pemphigoid, and lichen planus. Autoantigens and modified versions thereof are clustered at the cell surface blebs of apoptotic keratinocytes (8, 18, 19). Apoptotic surface blebs present autoantigens and likely release modified versions of them to circulation (18–24). It has been suggested that the release of modified autoantigens from apoptotic bodies could be the immunizing event that mediates systemic autoimmune responses leading to SLE and scleroderma (18, 24). Finally, phosphorylation has been reported to be a major modification that autoantigens undergo in apoptotic keratinocytes (19).

Although not being limited to an exact mechanism, we propose, in light of all of the above data, that GPBP/GPBPΔ26 likely represent two different strategies to regulate a kinase that is involved in the phosphorylation-dependent folding process of specific self-components. In certain tissues, the expression of the more active GPBP could serve to generate misfolded substrates as a part of an apoptotic-dependent strategy for desired cell removal, e.g. corneum stratum of the epidermis. Ectopic and/or augmented expression of GPBP could release an excess of altered antigens and engage the immune system in a more general autoimmune response as occurs in SLE or scleroderma. In other locations, e.g. the human glomerular and alveolar basement membranes, the presence of the active GPBP isoform may be required for efficient phosphorylation-mediated collagen IV assembly. An augmented expression of the alternative α3(IV)NC1 products alone or with increased levels of GPBP could result in the generation of α3(IV)NC1 conformers for which the immune system has not established a tolerance. Upon assembly, the altered conformers could engage the immune system in a tissue-restricted autoimmune response as in GP disease.

The substrate condition of the α3(IV)NC1 domain for GPBP (4) along with the presence of GPBP associated with the glomerular and alveolar basement membranes seem to predispose humans to undergo GP disease. Similarly, autoantigens and GPBP co-localize in the apoptotic bleb, and recombinant proteins representing autoantigens in SLE (P1 ribosomal phospho-protein and Sm-D1 small nuclear ribonucleoproteins) or in dermatomyositis (histidyl-tRNA synthetase) are in vitro substrates of GPBP (not shown). It is therefore likely that GPBP generates modified versions of the autoantigens which when released from the apoptotic bodies mediate the autoimmune responses in SLE or in dermatomyositis.

Although further studies are needed to define precisely the biological role of GPBP/GPBPΔ26, the low expression of GPBP in cancer cell lines (Fig. 2) and its high expression in the apoptotic bodies suggest that GPBP is involved in signaling pathways induced during programmed cell death. As discussed, GPBP expression could be up-regulated during autoimmune pathogenesis and mediate the immune response. Conversely, down-regulation of GPBP during cell transformation would prevent an autoimmune attack on the transformed cells during tumor growth.

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Goodpasture Antigen-binding Protein, the Kinase That Phosphorylates the Goodpasture Antigen, Is an Alternatively Spliced Variant Implicated in Autoimmune Pathogenesis

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