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Upregulation of the RAS-GTPase Activating Protein (GAP)-Binding Protein (G3BP) in Proliferating RPE Cells

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Abstract Cultured human retinal pigment epithelial (RPE) cells of different passages (P0 and P3) were used as a model system to examine changes in gene expression in proliferating RPE cells by polymerase chain reaction (PCR)-based differential expressed mRNA analysis (DEmRNA-PCR). DEmRNA-PCR showed enhanced expression of a specific RNA in P3 compared with P0. Sequence alignment displayed its identity with the 3′-end of the coding sequence of the human RAS-GTPase activating protein (GAP)-binding protein (G3BP). Confirmation of the induced expression of G3BP was performed by gene-specific reverse transcription-polymerase chain reaction (RT-PCR) of freshly prepared human RPE cells and of cultured cells of P0, P3 and P8 and by immunohistochemistry of cultivated retinal pigment epithelial cells in an artificial lesion assay. The expression of G3BP mRNA increased with the number of passages. G3BP protein expression increased in cells repopulating the artificial lesion. DEmRNA-PCR in RPE cells with subsequent sequence analysis led to the characterization of dedifferentiation- and proliferation-dependent expression of a previously undetected gene product in cultured RPE cells with a possible role in modifying signal transduction responses that may have implications on the treatment of proliferative vitreoretinopathy. J. Cell. Biochem. 74:194–201, 1999.

Key words: differential expressed mRNA reverse transcription-polymerase chain reaction; Ras-GTPase activating protein (GAP)-binding protein; G3BP; proliferative vitreoretinopathy; PVR

Proliferative vitreoretinopathy (PVR) remains the dominant cause for failure of retinal reattachment surgery in the majority of cases. PVR is characterized by dedifferentiation and proliferation of different cells, including fibroblasts, glial cells, macrophages, and retinal pigment epithelial (RPE) cells at the vitreoretinal interface, leading to extracellular membrane formation. Contraction of the membrane with subsequent traction on the retina leads to retinal detachment and severe impairment of vision [Vinores et al., 1990]. Many investigators have focused on the role of RPE cells in PVR, as these cells are frequently observed in surgically removed epiretinal membranes [Machemer and Laqua, 1975; Wiedemann, 1992]. The RPE is a differentiated and mitotically inactive monolayer between sensory retina and choriocapillaris in normal physiological conditions. But RPE cells lose epithelial characteristics and changes to a mesenchymal phenotype and proliferate when transferred into cell culture [Bryan and Campochiaro, 1986; Grisanti and Guidry, 1995]. Therefore, cultured RPE cells have been considered a valid model with which to study dedifferentiation and proliferation of RPE cells [Campochiaro, 1993; Abe et al., 1996]. The molecular mechanism of these changes is not fully understood. Recently, alteration of gene expression has been demonstrated in this model system by showing enhanced expression of the microtubuli-associated protein 1B in cultured RPE cells [Esser et al., 1997]. In this study, we

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further investigated the changes in RPE gene expression during cell culture to achieve new insights into the molecular events in RPE cell proliferation, using polymerase chain reaction (PCR)-based differential expressed mRNA analysis (DEmRNA-PCR)[Diachenko et al., 1986; Kociok et al., 1998].

MATERIALS AND METHODS

Materials

Minimal essential medium eagle (MEM-D-Val, D-valine substituted for L-valine), TRI Reagent, bovine serum albumin (BSA), rabbit serum, anti-pancytokeratin antibodies, nonimmune mouse IgG, and alkaline phosphatase (ALP)-conjugated antimouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Amphotericin B was obtained from vonHeyden (München, Germany) and Gentamycin from Merck (Darmstadt, Germany). Tissue culture flasks, four-compartment slide chambers (Nunc), fetal calf serum (FCS), trypsin/EDTA-solution and Superscript Preamplification System for First Strand cDNA Synthesis were purchased from Life Technologies (Eggenstein, Germany). Deoxyribonucleoside triphosphates (dNTPs) were from Boehringer Mannheim (Mannheim, Germany). The cloned thermostable DNA polymerase from *Thermus brockianus* (F500) PrimeZyme LE was obtained from Biometra (Göttingen, Germany). The mice anti-G3BP antiserum was a gift from Rhône-Poulenc Rorer (France). The Fast Red stain was purchased from Dako (Carpinteria, CA).

Cell Cultures

RPE were cultured from human donor eyes for keratoplasty within 2 h after death or from porcine eyes provided by the local abattoir. Briefly, RPE cells were harvested by trypsinizing the cells for 30 min with 0.2% trypsin containing 0.5 mM ethylendiaminetetraacetic acid (EDTA). The cells were centrifuged and seeded in culture flasks containing MEM-D-Val medium, supplemented with 15% FCS, 50 µg/ml gentamycin, and 2.5 µg/ml amphotericin. Phase-contrast examination of living cells was performed using a photomicroscope. RPE cell origin was confirmed by positive cytokeratin immunocytochemical analysis. The cells were kept in primary culture (P0) for approximately 3 weeks until confluence was reached. Subsequent passaging was performed as a dissociated single-cell suspension by trypsinization at similar time intervals. Cells were passaged or harvested for the experiments at the point of confluence. Five independent cell lines from donors aged 51–68 years were used for the experiments.

Differential Expressed mRNA PCR Analysis

Total RNA was prepared from freshly isolated human RPE cells (eye) and from cultured human RPE cells of primary culture (P0) and passage P3 and P8, using TRI Reagent. Total RNA concentration was determined spectrophotometrically ($A_{260}/A_{280} > 1.6$). Differential expressed mRNA PCR analysis (DEmRNA-PCR) was performed according to Kociok et al. [1998] using the arbitrary primers P10 and T8 described therein. Briefly, the reverse transcription was performed in parallel on 0.5 µg and 1.5 µg total mRNA from human RPE cells of passage P0 and P3, using the Superscript Preamplification System for First Strand cDNA Synthesis with oligo$(dT)_{12–18}$ primers. After heat inactivation and RNase H digestion, the first-strand synthesis mixture was diluted to a concentration of 5 ng/µl and 20 ng/µl with TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), respectively, relative to the total RNA put into the reaction. Diluted complementary DNA (cDNA, 2 µl) was mixed with 38 µl of PCR mixture for a final reaction solution containing 50 mM Tris-HCl (pH 9.0 at 25°C), 15 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 50 µM of each dNTP, 1 µM of primer P10 (5′-ATT AAC CCT CAC TAA AGC ACC GTC C-3′) and T8 (5′-CAT TAT GCT GAG TGA TAT CTT TTT TTT TGC-′) and 1 U of PrimeZyme LE. Thermocycling was performed in a PTC-100 thermocycler (MJ Research, Watertown, MA), using hotstart conditions, that is, heating of the mixture without DNA polymerase to 98°C for 5 min and adding DNA polymerase at 80°C. After hotstart, we used the following PCR cycle parameters: 1 cycle of 94°C for 5 min; 40°C for 5 min; and 72°C for 5 min; 2 cycles of 94°C for 2 min; 40°C for 5 min; and 72°C for 5 min; 29 cycles of 94°C for 1 min; 60°C for 1 min; and 72°C for 2 min, followed by a final extension at 72°C for 7 min. A total of 4 µl of the amplification reactions was run on a 0.4-mm nondenaturing 5% polyacrylamide gel, and amplified products were visualized by silver staining [Bassam et al., 1991]. Bands with different levels of intensity between the passages were selected

for subsequent reamplification and sequence analysis.

The band of interest (P10T801), with enhanced expression in P3, was cut out of the gel, placed in a PCR tube containing 50 µl of TE buffer, and boiled for 5 min. The eluted DNA in one microliter was reamplified in 50 µl, using the original primer pair (P10 and T8) and the previously described reaction mixture, but with 2 mM $MgCl_2$, 0.1 mM of each dNTP, and 0.2 µM of each primer. The following PCR cycle parameters were used for reamplification: hot start; 30 cycles of 94°C for 1 min; 60°C for 1 min; 72°C for 1 min, followed by a final extension at 72°C for 10 min. The reamplified samples were directly sequenced by a modification of the dideoxy chain termination method [Sanger et al., 1977], using the original primers.

Gene-Specific PCR

Using the primer analysis software OLIGO 4.1 (National Biosciences, Plymouth, MN), we selected specific primers for human RAS-GTPase activating protein (GAP)-binding protein (G3BP; AC: U32519): G3BPUp, pos. 720: 5′-CAG AAC CAG AAC AAG AAC CT-3′ and G3BPDo, pos. 1257: 5′-AAA CAC AAC AAA ACC AAA AT-3′, human β_2-microglobulin (B2MG, AC: V00567): B2MGLUP, pos. 77, 5′-CAG AGA ATG GAA AGT CAA AT-3′ and B2MGLDO, pos. 384, 5′-AAA AAG CAA GCA AGC AGA AT-3′, and human RPE65 (AC: U18991): HRPE6501UP, pos. 846, 5′-CCT TTC TTC ATG GAG TCT TTG-3′ and HRPE6502Do, pos. 1215, 5′-ATT GCA GTG GCA GTT GTA TTG 3′. Aliquots of the diluted cDNA of freshly harvested human RPE cells, human cultured RPE from P0, P3, and P8, corresponding to 62.5 ng, 12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 20 pg, and 4 pg initially used total RNA, were mixed with the above-mentioned PCR mixture, but with 0.2 mM of each dNTP and 0.2 µM of each specific primer. Using this fivefold dilution ensures that at least one of the dilutions lie within the range of linear progression of the reaction for the used number of cycles. For gene-specific amplification, we used the following PCR cycle parameters: hot start, 40 cycles of 94°C for 1 min; 53°C for G3BP, 50°C for B2MG, and 52°C for RPE65 for 1 min; and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplified DNA fragments had the expected length of 557 bp for G3BP, 327 bp for B2MG, and 390 bp for RPE65. No bands could be detected when non-reverse-transcribed RNA were used as template for PCR.

Semiquantitative Determination of G3BP mRNA Expression

For semiquantitative determination of G3BP mRNA expression in the eye and in cultured RPE cells of passages 0, 3, and 8, three (eye) and four independent preparations out of five cell lines were analyzed by RT-PCR. Photographs of ethidium bromide-stained gels of G3BP- and B2MG-specific RT-PCR were scanned and the band density determined using a densitometer (PDI, New York, NY) with Quantity One software. Within the range of linear progression of the PCR, the band density of G3BP was divided through the band density of B2MG. The mean density quotient of P0 RPE cells was defined as 100%, and the mean relative densities of G3BP expression in freshly harvested and passaged RPE cells (normalized to P0) were displayed graphically (see Fig. 3).

G3BP Immunocytochemical Analysis

Monolayers of porcine RPE cells were established by seeding isolated RPE cells on glass four-chamber slides. This primary cultures displayed a differentiated cobblestone morphology with intense pigmentation. Lesions were made in the confluent layer by performing a 2-mm-wide linear scratch. After 72 h, RPE cells at the edge of the lesion had migrated into the empty space. The cells were then washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at 4°C.

The RPE cells were incubated with blocking buffer PBS, 20% normal rabbit serum, and 5% BSA) for 1 h in a moist chamber at room temperature. Immunocytochemical analysis was accomplished using a mice anti-G3BP serum, with subsequent visualization by ALP-conjugated antimouse IgG and Fast Red stain. Negative controls were performed by changing the anti-G3BP antiserum with nonimmune mouse IgG.

RESULTS

As expected, using the nonspecific primer combination P10 and T8 in the DEmRNA-PCR analysis of human RPE cells of primary culture (P0) and P3 resulted in the appearance of more than 50 bands by silver staining. This primer depending banding pattern were reproducible within a cell line. Increased amplification of a

band referred to as P10T801 at approximately 840 bp was consistently observed in third-passage cells as compared with primary cultures (Fig. 1A) in all three investigated independent RPE cell populations. Reamplification of the eluted cDNA from this excised band, using the same primer combination, confirmed the PCR fragment of approximately 840 bp (Fig. 1B). Sequence alignment with EMBL human gene bank sequences with 181 sequenced bases of P10T801 showed its identity with the 3′-end of the complete coding sequence of human G3BP. Now using gene-specific primers for G3BP, we confirmed expression of G3BP mRNA in human RPE cells and its upregulation from freshly prepared RPE cells, over RPE cells from P0 and P3 to P8 (Fig. 2A). The specific band for the primer combination used here has a length of 557 bp. Whereas for RT-PCR of RPE cells from the eye and P0 a cDNA input corresponding to 500 pg total RNA is necessary to obtain a clear band (Fig. 2A, lane 4), only 100 pg is necessary for P3 (Fig. 2A, lane 5) and 100–20 pg for P8 (Fig. 2A, lanes 5 and 6). cDNA fragments of β_2-microglobulin (B2MG) (327 bp) were amplified as an internal standard (Fig. 2B). Normalizing the level of intensity of G3BP to B2MG showed an approximately twofold increase of G3BP band intensity from freshly prepared RPE cells (56 ± 9,2%) to P0 (defined as 100%), an additional twofold increase from P0 to P3. 195 ± 61,6%) and a further increase from P3 to P8 (247 ± 78,5%) (Fig. 3). A cDNA fragment of the RPE cell-specific gene RPE65 (390 bp) was amplified from freshly prepared RPE cells and from P8, as a known marker for downregulated gene expression during cell culture [Nicoletti et al., 1995; Hamel et al., 1993]. Indeed, the high intensity level of RPE65-specific mRNA in freshly prepared RPE cells was markedly reduced in P8 cells (Fig. 2C).

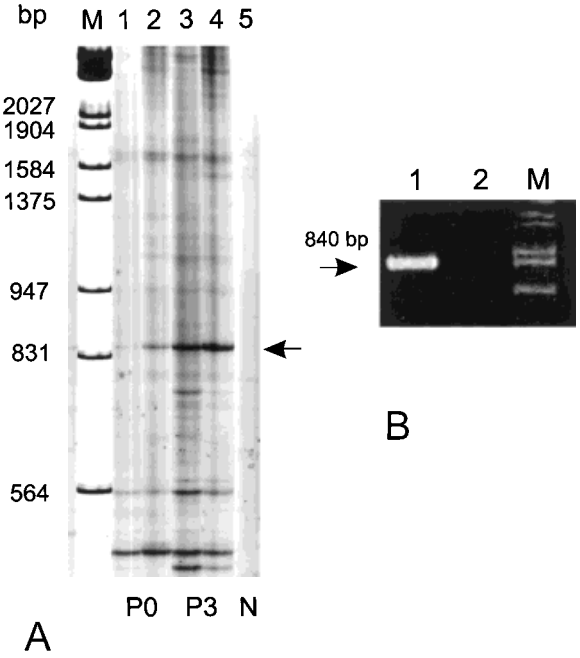

Fig. 1. Identification of RAS-GTPase activating protein (GAP)-binding protein (G3BP) mRNA as induced by serially passaged human retinal pigment epithelial (RPE) cells. **A:** DEmRNA-PCR, performed as described under Materials and Methods, reveal multiple bands of cDNA fragments in P0 and P3 cultured human RPE cells. As indicated, 0 µg (as negative control, **lane 5**), 0.5 µg (**lanes 1, 3**), and 1.5 µg (**lanes 2, 4**) total RNA were used in the reverse transcription (RT) reactions. M, the ladder of DNA size markers. A band at approximately 840 bp (arrow) shows an elevated band intensity in P3 compared with P0. **B:** Using the same primers, reamplification of complementary DNA (cDNA) eluted from this band confirms amplification of an 840-bp polymerase chain reaction (PCR) fragment (**lane 1**). **Lane 2**, PCR control with no DNA template. DNA size markers in **lane M**, correspond to 1584, 1375, 947, 831, and 564 bp.

Next, we investigated the expression of G3BP in cultured RPE cells at the protein level. For this purpose, easily available porcine RPE cells were used for better standardization of the cultures and improved reproducibility of the experiments. In order to compare cultured P0 cells in quiescent as well as activated stages, we used the monolayer artificial wound assay outlined in the Material and Method section. Within 72 h after the scratch was imposed, cells at the wound edge repopulated the injured area of the monolayer. G3BP was immunocytochemically detected at a low level in the primary monolayers of RPE cells and at a high level in cells at the edge and, notably, in repopulating cells within the damaged area of the monolayer (Fig. 4A–C). No staining was observed in the negative controls when isotype nonimmune mouse IgG was substituted for the G3BP mouse serum (Fig. 4D).

DISCUSSION

The retinal pigment epithelium (RPE) forms a highly differentiated and mitotically inactive monolayer of polarized cells situated between the photoreceptors and the choroidal capillaris [Zinn and Benjamin-Henkind, 1979]. Through its many essential functions, including control of transport of ions, nutrients, macromolecules and fluid between the blood supply and the photoreceptors, phagocytosing the shed tips of photoreceptor outer segments, and regenera-

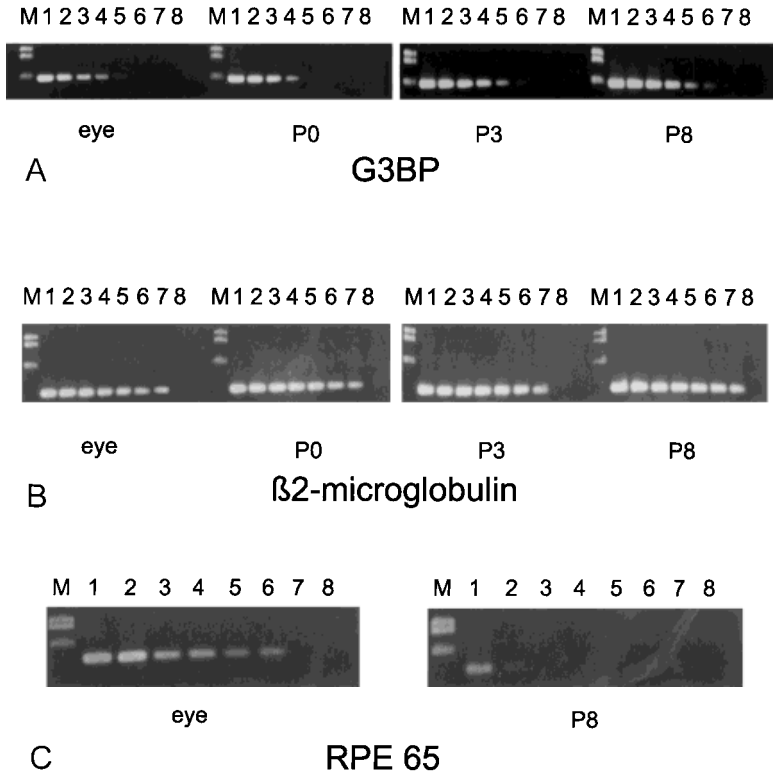

Fig. 2. Upregulation of RAS-GTPase activating protein (GAP)-binding protein (G3BP) mRNA in freshly harvested and serially passaged human retinal pigment epithelial (RPE) cells. RNA was prepared from freshly harvested cells (eye), and passaged RPE cells (P0, P3, and P8). Reverse transcription-polymerase chain reaction (RT-PCR) for G3BP (A), β_2-microglobulin (B2MG) (B), and the RPE-specific gene RPE65 (C) was performed as described under Materials and Methods. **A,C: Lanes 1–8**, correspond to a cDNA input into the PCR corresponding to 62.5 ng, 12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 20 pg, 4 pg, and 0 ng (PCR control) total RNA in the reverse transcription sample. **B: Lanes 1–8**, correspond to an additional fivefold diluted cDNA input (12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 20 pg, 4 pg, 0,8 pg, and 0 ng (PCR control) total RNA. M, the ladder of DNA size markers (947, 831, and 564 bp). A: Level of the G3BP-specific PCR fragment (557 bp) increases from freshly harvested to serially passaged human RPE cells. B: Level of the B2MG-specific PCR fragment (327 bp) remains constant in freshly harvested and serially passaged human RPE cells. C: Level of the RPE-specific PCR fragment RPE65 (390 bp) decreases clearly in P8 human RPE cells compared with freshly harvested cells.

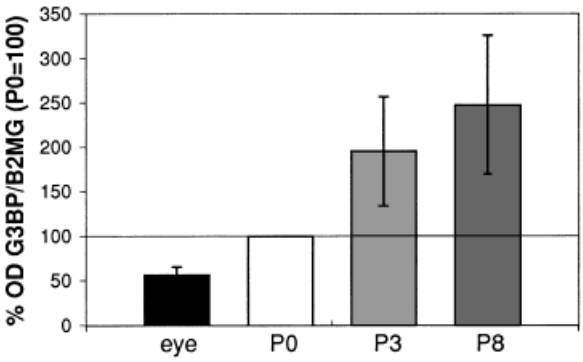

Fig. 3. Increasing RAS-GTPase activating protein (GAP)-binding protein (G3BP) mRNA expression in freshly harvested and serially passaged human retinal pigment epithelial (RPE) cells. The quotient of G3BP expression to B2MG expression was determined as described under Materials and Methods. The relative G3BP mRNA expression of P0 RPE cells was defined as 100%. Relative G3BP mRNA expression in the eye and in passage 0, 3, and 8 is displayed graphically (with standard deviation bars). Relative G3BP mRNA expression increases from 56% in freshly harvested RPE cells to nearly 250% in P8 RPE cells.

tion of the visual pigments, RPE cells are vital in maintaining the visual process [Zinn and Marmor, 1979; Marmor and Wolfensberger, 1998]. It is widely accepted that proliferating RPE cells in pathologic conditions play an essential role in the formation of proliferative vitreoretinopathy (PVR) [Glaser and Lemor, 1994; Campochiaro, 1997]. In addition to studying RPE cell responses to soluble proteins, e.g., growth factors [Leschey et al., 1990; Campochiaro, 1993], advances in molecular biology now offer the opportunity of identifying known or novel genes, previously unassociated with the process examined [Hunt et al., 1998]. Proliferating cultured RPE cells have been considered a valid model with which to study dedifferentiation and proliferation of RPE cells [Campochiaro, 1993]. Using the same technique as in this study (DEmRNA-PCR analysis), we recently demonstrated in this model that the microtubule-associated protein 1B plays an important role in the phenotypic alterations of RPE cells, responding to distinct environmental stimuli [Esser et al., 1997].

In this study, we demonstrate that the relative level of mRNA expression of G3BP is specifically enhanced nearly threefold from freshly prepared RPE cells to P8, normalized to the mRNA expression of the gene for the β-chain of major histocompatibility complex class I molecule, β_2-microglobulin (B2MG). In contrast to the increased expression of G3BP is the decreased mRNA expression of the RPE cell-specific RPE65 gene. The function of RPE65 is

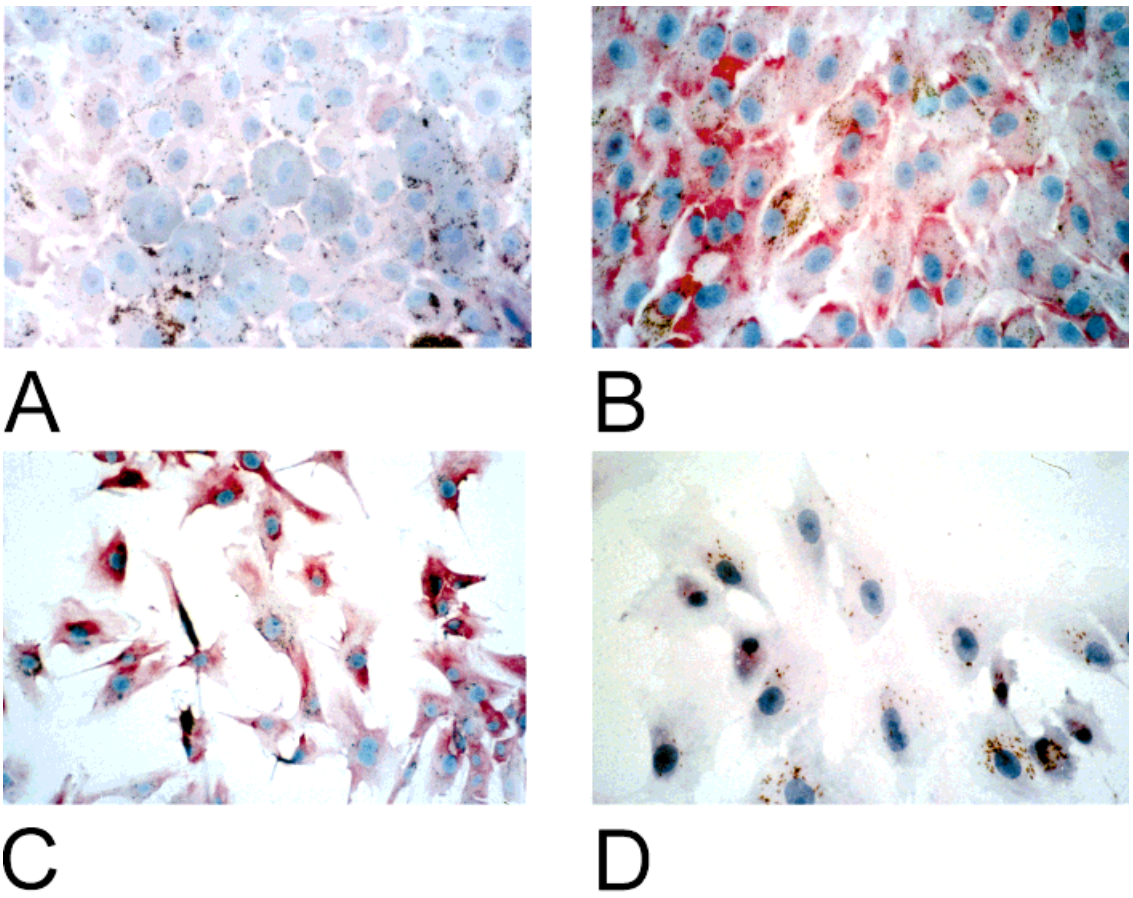

Fig. 4. Increasing RAS-GTPase activating protein (GAP)-binding protein (G3BP) expression in proliferating cultured porcine retinal pigment epithelial (RPE) cells repopulating an artificial wound in a monolayer. Immunocytochemical analysis of G3BP on a primary monolayer with artificial set lesions was performed as described under Materials and Methods. **A:** Fast Red staining with the mouse anti-G3BP serum shows a weak G3BP expression in primary monolayer of differentiated RPE cells with typical cobblestone morphology and intense pigmentation. **B:** G3BP expression is increased in cells near the edge of the lesion in the monolayer with beginning changing morphological phenotype. **C:** Within the damaged area, G3BP expression is notably increased in proliferating cells with extreme changed morphological phenotype. **D:** No staining occurs with the nonimmune mouse serum (control) on repopulating cells with extreme changed morphological phenotype. A–C: ×200; D: ×400.

unclear. It has been proposed that RPE65 might encode for retinol isomerase, one of the key enzyme of the retinoid metabolism of the RPE cell [Redmond et al., 1997]. This proposed function makes a decreased expression of RPE65 in cultured RPE cells understandable. The constant mRNA expression of the B2MG gene, in addition to the simultaneous decrease of RPE65 mRNA expression, confirms that the increased G3BP expression is specific and not due to a general increase of expression because of progressive cell activity on in vitro conditions. Our immunocytochemical analysis in the artificial lesion assay further show that G3BP's expression at the protein level increase from primary monolayers of RPE cells to cells at the edge and, notably, in repopulating cells within the damaged area of the monolayer. As previously demonstrated [Grisanti and Guidry, 1995], these proliferating cells lose the typical epithelial cobblestone morphology toward an elongated fibroblastic cell type. Similar morphologic changes have been observed when RPE cells proliferate in the vitreous in vivo, as occurs in PVR [Hunt et al., 1998]. Future immunocytochemical studies in surgically removed epiretinal membranes from patients with PVR will show whether the expression of G3BP is also upregulated in proliferating RPE cells in vivo.

G3BP was recently purified, cloned, and sequenced [Parker et al., 1996]. The precise function of G3BP remains known. G3BP, a 466-

amino acid protein, is located mainly in the cytosol and binds to the SH3 domain of GAP [Briggs et al., 1995], which is essential for the signal transduction function of Ras [Yang et al., 1994; Bryant et al., 1996; Pomerance et al., 1996]; in addition to being a negative regulator of Ras, GAP may also represent a downstream target of Ras [Duchesne et al., 1993]. G3BP is the first protein described that binds effectively to GAP SH3. The coimmunprecipitation of G3BP and GAP occurs only when cells are in a proliferating state, suggesting a recruitment of a GAP-G3BP complex when Ras is in its activated conformation [Parker et al., 1996]. This finding fits well with our finding that the expression of G3BP is upregulated in dedifferentiated and proliferating RPE cells.

G3BP shares several features with heterogeneous nuclear RNA-binding proteins, including ribonucleoprotein (RNP) motifs RNP1 and RNP2, an RG-rich domain, and acidic sequences [Parker et al., 1996]. It was suggested that G3BP may represent an adapter protein, connecting RNAs to upstream signaling molecules through a tight binding to SH3 motifs thus affecting the stability and the translational efficiency of mRNAs of specific proteins required for Ras-dependent responses [Tocque et al., 1997] as PolyA-binding protein and the eukaryotic translation initiation factor 4B do for the 3′-end and 5′-end of mRNAs.

Very recently, phosphorylation-dependent endoribonuclease activity of G3BP was demonstrated that ascribe G3BP as a link between a RasGAP-mediated signaling pathway and RNA turnover. G3BP may be a component of an mRNA degradation system that controls normal cell growth and differentiation [Gallouzi et al., 1998]. Again, these proposed functions of G3BP fit well with its changed regulation in proliferating RPE cells.

In light of the proposed functions of G3BP, its increased mRNA and protein expression in our models of proliferating RPE cells suggest that G3BP plays a role in the dedifferentiation process of RPE cells, responding to environmental stimuli. Quite obviously other genes are upregulated as well and in each case a causal relationship in addition to a mere correlation has to be shown. Indeed, a specific upregulation of an other gene in this system was shown before for MAP1B [Esser et al., 1997]. However, whereas MAP1B may be directly responsible for the phenotypic alteration of RPE cells by determining the structure of the microtubules, G3BP in RPE cells may modulate the Ras-mediated signal transduction of cytokines in normal physiological as well as pathological situations, like proliferative vitreoretinal diseases. Thus, although we have found only a correlation between increased G3BP expression and proliferating cultured RPE cells, it is attractive to speculate that G3BP could become an attractive target for therapeutic intervention in the future by influencing the action of many cytokines at the same time if a causal relationship could be established.

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