The integrin α5β1 regulates chondrocyte hypertrophic differentiation induced by GTP-bound transglutaminase 2

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The non-standard abbreviations used are: TG2, transglutaminase 2; Ct, cycle threshold

Abstract

Soluble GTP-bound transglutaminase 2 (TG2) induces hypertrophic differentiation in chondrocyte cultures in a β1 integrin-dependent fashion. β1 integrin subfamily consists of 12 heterodimers with 12 different α subunits and a β1 subunit. To identify the specific integrin heterodimer(s) responsible for this process, we specifically blocked individual β1 integrins on the CH-8 immortalized human chondrocytes during hypertrophic differentiation. Blockade of α5β1 inhibited matrix metalloproteinase 13 (MMP-13), type X collagen expression, alkaline phosphatase activity, and matrix calcification by 30-50 %, associated with weak effects of anti-α3β1 and -α4β1. Anti-α1β1, -α2β1 and -α6β1 had no effect. To examine whether the dominant effect of integrin α5β1 was due to a direct interaction with TG2, we incubated the chondrocytic cells on plates coated with GTP-bound TG2. The immobilized GTP-bound TG2 induced hypertrophic differentiation to the same extent as the soluble GTP-bound TG2, which was also inhibited by anti-α5β1. CH-8 cells grown on plates coated with GTP-bound TG2 demonstrated adherence associated with focal adhesion kinase phosphorylation. These properties were inhibited by anti-α5β1. Furthermore, engagement of α5β1 on CH-8 cells via anti-α5β1 antibody did, in fact, induce differentiation. Although CH-8 cells adhered to GTP-free TG2 via integrin α5β1, the cells failed to undergo hypertrophic differentiation. Thus, integrin α5β1 is critical for the chondrocyte hypertrophic differentiation induced by GTP-bound TG2, and this induction is ligand-dependent.

Key words

Integrin; Transglutaminase 2; Chondrocyte; Hypertrophy; Differentiation

Introduction

Endochondral bone formation requires the activation of resting chondrocytes to a terminal hypertrophic state (Erlebacher et al., 1995) (Karsenty and Wagner, 2002). A similar pathway is responsible for pathological articular cartilage calcification (von der Mark et al., 1992) (Goldring, 2000). This process is accompanied by release of mineralization-competent calcium-rich vesicles, rapid matrix remodeling, and expression of mineralization-related genes, including annexin II, annexin V, annexin VI, alkaline phosphatase (ALP), matrix metalloproteinase 13 (MMP-13), and type X collagen (Col X). One of the features common to hypertrophic chondrocytes in growth plate and in articular cartilage is the upregulation of the enzyme TG2 (EC 2.3.2.13). Transglutaminase is a widely distributed intracellular and extracellular calcium-dependent enzyme, which catalyzes the polymerization of substrate proteins by creating isopeptide cross-links between glutamine and lysine residues. TG2 has been suggested to be involved in matrix maturation and stabilization through these cross-links. This enzyme is unique among the human transglutaminase family due to its dual function as a transglutaminase and a GTPase/ATPase. We have recently shown that this enzyme is also a disulfide-isomerase (Hasegawa et al., 2003), and others have described protein kinase activity (Mishra and Murphy, 2004) as well.

Recently, soluble extracellular TG2 has been reported to induce chondrocyte hypertrophic differentiation (Johnson and Terkeltaub, 2005). This function is markedly augmented by GTP-binding and is β1 integrin-dependent. Integrins are αβ heterodimeric transmembrane proteins that are comprised of 18 α and 8 β subunits as part of 24 heterodimers (Hynes, 2002). Of the twelve integrins that contain the β1 subunit, at least five (α1β1, α3β1, α4β1, α5β1 and α9β1) have been reported to be associated with (Akimov et al., 2000; Zemskov et al., 2006) or receptors for (Takahashi et al., 2000) TG2. The aim of this study was to identify which chondrocyte β1 integrin(s) mediates the hypertrophic

differentiation induced by GTP-bound TG2 with expectation that this will provide insight into the regulation of chondrocyte hypertrophic differentiation.

Results

Purified transglutaminase 2 from guinea pig liver induces human chondrocyte hypertrophic differentiation in a β1 integrin-dependent fashion.

HEK293 cell-derived recombinant human GTP-bound TG2 protein has been reported to induce chondrocyte hypertrophic differentiation with concomitant upregulation of Col X expression and matrix calcification (Johnson and Terkeltaub, 2005). To determine if the TG2 we purified from guinea pig liver could also induce hypertrophic differentiation, we incubated CH-8 cells in the presence or absence of GTP-bound TG2 and evaluated Col X expression and matrix calcification together with two other indicators of hypertrophic differentiation, MMP-13 expression and ALP activity. Expression of Col II and aggrecan were also measured as phenotypic markers of proliferating chondrocytes. After five days in culture, GTP-bound guinea pig TG2 induced a five-fold increase in MMP-13 expression and a two-fold increase in Col X expression (Fig 1A). ALP activity was increased three-fold, and matrix calcification was elevated more than 1.5-fold (Fig. 1B). In contrast, expression of Col II and aggrecan were decreased to nearly undetectable levels (Fig. 1A). All of these changes were completely inhibited following incubation with an integrin β1-blocking antibody (Fig. 1A, 1B). Thus, guinea pig TG2 induces chondrocyte hypertrophic differentiation in a β1 integrin-dependent manner.

Hypertrophic chondrocyte differentiation induced by soluble GTP-bound transglutaminase 2 is

mediated by integrins, α3β1, α4β1, and α5β1.

To determine which of the β1 integrins contribute to differentiation, we first assessed the expression of eleven α subunit partners of the β1 integrin subunit by flow cytometry using a panel of integrin antibodies or by quantitative RT-PCR. Flow cytometry indicated that CH-8 cells expressed the α1, α3, α4, α5, and α6 subunits. The α2 subunit was expressed at very low levels, and α9 was undetectable (Fig. 2A). RT-PCR revealed that the CH-8 cells expressed α7, α10, and α11 but not α8 (Fig. 2B, 2C). We then used blocking antibodies against the α1, α2, α3, α4, α5, and α6 subunits in the differentiation culture (blocking antibodies for α7, α10, and α11 were not available). Each antibody resulted in similar effects for each of the four measured indices of hypertrophic differentiation (Fig. 3). The anti-α5 antibody showed the most potent inhibitory effects followed by anti-α4 and anti-α3. The anti-α1, anti-α2, and anti-α6 antibodies produced only minimal effects (Fig. 3, light gray columns). The results of experiments with a variety of antibody combinations confirmed that the blocking of α5β1 was the most potent inhibitor of differentiation (Fig. 3, dark gray columns). Thus, α5β1 is the principal integrin responsible for these effects with probable minor contributions from both the α3 and α4 subunits. Although we cannot specifically exclude the possibility that integrins α7β1, α10β1, or α11β1 contribute to the hypertrophic differentiation, the effect appears minimal because the combination of anti-α3, anti-α4, and anti-α5 inhibited the increases of all four indices (Fig. 3) to the same degree as anti-β1 alone (Fig. 1A, 1B).

Immobilized GTP-bound transglutaminase 2 induces chondrocyte hypertrophic differentiation mediated by integrin α5β1.

Integrin signaling has generally been studied in response to immobilized ligands. To more directly examine the role of integrin ligation and signaling in chondrocyte hypertrophic differentiation

induced by GTP-bound TG2, we determined whether immobilized GTP-bound TG2 would also induce each of the differentiation markers used above. We seeded CH-8 cells on plates coated with free or GTP-bound TG2 and then examined induction of hypertrophic differentiation (Fig. 4). CH-8 cells differentiated on immobilized GTP-bound TG2. In addition, differentiation was inhibited by an anti-α5 antibody. The increases in MMP13, Col X, ALP, and matrix calcification were five-fold, two-fold, three-fold, and 1.5-fold, respectively. These effects were similar to those observed for soluble GTP-bound TG2 (Fig. 1). In contrast, the immobilized free TG2 did not affect any of these differentiation markers.

Cell adhesion and phosphorylation of FAK is mediated by integrin α5β1.

To examine the involvement of integrin α5β1 in the immobilized GTP-bound TG2-induced differentiation, we assessed cell adhesion and phosphorylation of FAK, a signaling molecule immediately downstream of integrin signaling. Cell adhesion in CH-8 cells that had adhered well to plates coated with GTP-bound TG2 was largely inhibited by antibodies to α5 (Fig. 5). As we observed for differentiation in response to soluble GTP-bound TG2, anti-α3 or anti-α4 only weakly inhibited adhesion. The addition of anti-α3, anti–α4, and anti-α5 together inhibited adhesion to the same extent as anti-β1. Next, phosphorylation of FAK in CH-8 cells was assessed via western blot (Fig. 6). Phosphorylation was very weak in non-adherent cells; however, after 10 or 30 minutes of adhesion, the phosphorylation of FAK was apparent and was reduced by incubation of cells with anti-α5β1. This reduction in phosphorylation by the α5β1 antibody was calculated too be approximately 75 % of the phosphorylation of 30-minute sample. These results indicate that the chondrocytic cell line CH-8 adherence to immobilized GTP-bound TG2 was mediated by integrin α5β1. Furthermore, adhesion dependent signal was initiated through α5β1.

Engagement of integrin α5β1 with antibody reproduces the effects of GTP-bound transglutaminase 2 on chondrocyte hypertrophic differentiation.

To determine whether clustering of α5β1 might induce chondrocyte hypertrophic differentiation, CH-8 cells were seeded on plates coated with an anti-α5 antibody at concentrations of 1, 10, 20 and 50 μg/ml. Each of the four indicators (MMP-13 expression, Col X expression, ALP activity, and matrix calcification) was increased in a dose-dependent fashion (Fig. 7). Col X expression and ALP activity were both increased by 2-3-fold, and MMP-13 expression and matrix calcification were increased about two-fold at the highest concentration of antibody. The increases in these endpoints were very similar to those induced by GTP-bound TG2 except for MMP-13 (two-fold increase), which was smaller than the change induced by GTP-bound TG2 (five-six fold increase). Although the induction pattern was not identical to that produced by the interaction of the integrin with the GTP-bound TG2, engagement of the α5β1 integrin with its specific antibody clearly induced the hypertrophic differentiation.

CH-8 cells adhered to GTP-free transglutaminase 2 through α5β1 but failed to induce the hypertrophic differentiation.

As shown in Figure 4, immobilized free TG2 did not induce chondrocyte hypertrophic differentiation; however, we have previously reported that GTP-free TG2 is, in fact, a ligand for integrin α5β1 on the human colon cancer cell line SW480 (Takahashi et al., 2000). Therefore, we tested whether integrin α5β1 is involved in mediating cell adhesion to GTP-free TG2 in CH-8 cells. CH-8 cells adhered to GTP-free TG2, and this adherence was inhibited largely by anti-α5 (Fig. 8A). These results were similar to those with GTP-bound TG2 (Fig. 8A). CH-8 cells adhered to the GTP-free TG2

similarly to the GTP-bound TG2 at concentrations of 1-30 μg/ml (Fig. 8B).

We then added soluble GTP (0.25 to 250 μM) to the cultures of CH-8 cells that had been plated on GTP-free TG2. This addition did not induce differentiation as measured by the four endpoints (Fig. 9A). Soluble GTP was also added to cultures of CH-8 cells plated on immobilized anti-α5 antibody. Surprisingly, this addition did not produce an additive effect to the antibody-induced hypertrophic differentiation. These results indicated that GTP did not add second signal independently to that from integrin α5β1.

Discussion

TG2 has been reported to promote chondrocyte hypertrophic differentiation in TG2 knockout mice (Johnson et al., 2003). Two year after this report, the inductive effect of external GTP-bound TG2 on chondrocyte hypertrophic differentiation and the inhibitory effect of β1 integrin antibody on Col X expression were reported (Johnson and Terkeltaub, 2005). The results of the present study demonstrate that integrin α5β1 is responsible for this response. While soluble GTP-bound TG2 upregulated the four examined chondrocyte differentiation markers (MMP-13, Col X, ALP, and matrix calcification), the consistent and effective reduction of these markers by incubation of these cells with the anti-α5 antibody indicated that α5β1 contributes significantly to the differentiation process. As with soluble GTP-bound TG2, CH-8 cells plated on the immobilized GTP-bound TG2 underwent hypertrophic differentiation that was also blocked by anti-α5 antibody. These results further support the idea that integrin α5β1 plays an essential role in this differentiation pathway. This induction by immobilized GTP-bound TG2 was associated with cell adhesion and phosphorylation of pp125FAK, and these

cellular effects were also inhibited by the anti-α5 antibody. Furthermore, the role of α5β1 was confirmed by induction of the hypertrophic via artificial clustering of this receptor with immobilized antibody. This result demonstrated that downstream signaling from α5β1 induces chondrocyte hypertrophic differentiation.

The role of chondrocyte integrins in many biological processed have been chronicled recently in multiple reports (Shakibaei, 1998) (Cao et al., 1999) (Attur et al., 2000) (Millward-Sadler and Salter, 2004). The importance of integrins in chondrocytes has been confirmed by genetic engineering. Mice lacking integrin-linked kinase or β1 integrins in chondrocytes demonstrated poor proliferation of chondrocytes and chondrodysplasia (Grashoff et al., 2003) (Aszodi et al., 2003). The immortalized chondrocyte CH-8 expressed the β1-integrins, α1β1, α3β1, α4β1, α5β1, α6β1, α7β1, α10β1, and α11β1. In accordance with this integrin expression pattern, adult articular chondrocytes were found to consistently strongly express α1β1 and α5β1 (Loeser, 2000). In fact, α5β1 was found to be a predominant chondrocyte integrin (Salter et al., 1992). The predominant role of α5β1 in chondrocytes includes inflammatory response (Attur et al., 2000), survival signal (Pulai et al., 2002), and mechanotransduction (Millward-Sadler and Salter, 2004). Two previous reports support our hypothesis that α5β1 is critical for chondrocyte hypertrophic differentiation. In the first, the addition of RGD-peptide that binds to α5β1 into an *in vitro* endochondral ossification model resulted in an increase in ALP activity and chondrocyte enlargement (Yasuda et al., 1996). In the second, the injection of α5β1 antibody into the embryonic mouse wrist inhibited chondrocyte hypertrophic differentiation while over expression of α5β1 in chicken legs by injection of α5 and β1 cDNA induced chondrocyte hypertrophic differentiation (Garciadiego-Cázares et al., 2004). Taken together with the data presented here, these reports underscore the importance of integrins, especially α5β1, in chondrocyte biology and suggest direct involvement of α5β1 in chondrocyte hypertrophic differentiation.

The induction of chondrocyte hypertrophic differentiation by external TG2 does not require cross-linking activity or fibronectin binding (Johnson and Terkeltaub, 2005). GTP-binding, however, is required for soluble TG2 to induce differentiation, as seen in this study. Although we cannot explain the requirement for GTP binding, we excluded the possibility that differences in inducing integrin clustering by GTP-bound and GTP-free TG2 caused the differences in effects on chondrocyte differentiation by these two enzymes as found in the previous study (Johnson and Terkeltaub, 2005). Since the addition of GTP to cultures of CH-8 cells plated on immobilized GTP-free TG2 did not induce chondrocyte hypertrophy, we also excluded the possibility that GTP adds a second signal independently of the TG2-α5β1 interaction. In support of this conclusion, the addition of GTP to CH-8 cells plated on anti-α5β1 antibodies did not show obvious enhancing effects. Therefore, the direct binding of GTP is indispensable for TG2 to induce the hypertrophic differentiation. Although recent studies suggested that the GTPase activity of TG2 in the cytoplasm may be involved in regulation of cell motility (Kang et al., 2004; Stephens et al., 2004), the biological implications of GTP binding by external TG2 is unknown. Possible conformational changes and the resultant functional alterations on stability (Zemskov et al., 2006) and interaction with other molecules may be involved in the induction of hypertrophic differentiation.

In this report, we have clearly demonstrated that GTP-bound TG2-induced chondrocyte hypertrophic differentiation is mediated by integrin α5β1 in a ligand-dependent fashion. Identification of α5β1 integrin ligands capable of inducing chondrocyte hypertrophic differentiation would provide insight into the regulatory mechanism of endochondral bone formation and the medical control of osteoarthritis.

Experimental Procedures

***Reagents*-**Bovine serum albumin (BSA), L-ascorbic acid 2-phosphate, $MgCl_2$, GTP, Alizarin-Red S, and cetylpyridinium chloride were purchased from Sigma-Aldrich Japan (Tokyo, Japan). TG2 was purified from guinea pig liver as previously described (Takahashi et al., 2000). The following anti-human integrin monoclonal antibodies were purchased from Chemicon (Temecula, CA) and used for functional blocking or flow cytometry: FB12 against α1, P1E6 against α2, P1B5 against α3, P1H4 against α4, P1D6 against α5, GoH3 against α6, and P5D2 against β1. Fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies used for flow cytometry were AK7 against α2 subunit (Chemicon), HP2/1 against α4, and SAM-1 against α5 (Beckman Coulter, Fullerton, CA). Anti-α9β1 monoclonal antibody Y9A2 was obtained from Dr. Dean Sheppard (UCSF, San Francisco, CA). Fluorescently labeled goat anti-mouse and anti-rat IgG were from Invitrogen. Rabbit polyclonal anti-FAK antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phosphorylated FAK (pY397) was obtained from Invitrogen (Grand Island, NY).

***Cell line and culture*-**A Simian virus 40 immortalized clone of normal human knee articular chondrocytes (CH-8 cells) was kindly provided by Dr. Shin Aizawa (Nihon University, Tokyo, Japan) and maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Biological Industries, Haemek, Israel), 1% glutamine, 100 units/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml amphotericin B (Nacalai Tesque, Kyoto, Japan), and 50 μg/ml of ascorbic acid (Sigma-Aldrich Japan) at 37 °C as described (Yoshimatsu et al., 2001). The chondrocyte phenotype was verified in the CH-8 cells throughout this study by reverse transcriptase-PCR for expression of type II collagen (Col II) and aggrecan. Differentiation studies were

performed by incubating the cells in DMEM high glucose as described above except for only 1 % fetal calf serum for five days unless otherwise stated. TG2 was treated to generate magnesium nucleotide complexes (Lai et al., 1998). In brief, 1 mM $MgCl_2$ was added to 25 μM GTP and incubated on ice for 30 min with 0.1 μg of the guinea pig TG2**.**

Real-time RT-PCR -Total RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA) and reverse transcribed by the ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) using oligo-dT primers. TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes were used for the PCR step in an Applied Biosystems 7300 real-time PCR system (Foster City, CA, Assay ID for MMP-13, Hs00233992_m1; Col X, Hs00166657_m1; Col II, Hs00264051_m1; aggrecan Hs00202971_m1; integrin subunit α7 Hs00174397_m1, integrin subunit α8 Hs00233321_m1, integrin subunit α10 Hs00174623_m1 , integrin subunit α11 Hs00201927_m1). Primer sequences are not publicly available although their validity has been established by the manufacturer. The expression values were normalized against those from the GAPDH control from the same samples. Real-time PCR data were analyzed using the 7300 System software (version 1.2; Applied Biosystems). Ct (cycle threshold) value indicates the number of PCR cycles required to amplify the template to the level calculated automatically by the software.

Alkaline phosphatase (ALP) activity and matrix calcification assay-To measure ALP activity, 2 x 10^4 CH-8 cells were plated in 24-well plates and incubated as described above for five days. The cells were then washed with phosphate buffered saline (PBS) and lysed with 0.05% Triton X-100. ALP activity was measured by the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol using a Wako ALP kit (Wako Pure Chemical, Osaka, Japan). To quantify matrix calcification, the Alizarin red-S binding assay

was performed as described (Stanford et al., 1995). In brief, approximately 2×10^3 CH-8 cells were plated per well in 96-well plates in differentiation medium supplemented with 1 mM sodium phosphate. Cells were grown for five days. At the end of each experiment, cells were briefly rinsed with PBS followed by fixation in ice-cold 70% ethanol for one hour. Fixed cells were rinsed with water and stained for 10 min with 40 mM Alizarin red-S, pH 4.2, at room temperature. The cells were then rinsed with water four times followed by a 15-min wash with PBS. Then, 0.1 ml of 10% (wt/vol) cetylpyridinium chloride was added for 15 min to release the remaining calcium-bound Alizarin red-S. The Alizarin red-S concentration was determined by absorbance at 562 nm on a microplate reader (Molecular Device, Sunnyvale, CA). For both assays, the values were normalized to protein concentrations determined using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Flow cytometry-Cells were grown to 80 % confluence, detached with trypsin-EDTA (Invitrogen), washed with PBS (pH 7.4), and then incubated with goat serum for 10 min on ice. Cells were collected by centrifugation, exposed to saturating concentrations of antibodies raised against integrins α1 (FB12), α2 (AK7), α3 (P1B5), α4 (HP2/1), α5 (SAM1), α6 (GoH3), and α9β1 (Y9A2) in PBS for 10 min on ice. When the primary antibodies were not fluorescently labeled (α1, α3, α6, and α9β1), cells were incubated with secondary antibody for 20 min on ice. After cells were washed with PBS twice, expression of the integrins was then quantified on 10,000 cells with a FACS Calibur (BD, San Jose, CA).

Cell adhesion assays-Cell adhesion assays were performed as previously described (Yokosaki et al., 1994). Wells of polystyrene 96-well flat-bottom micro titer plates that were not tissue culture-treated (Nunc Inc., Naperville, IL) were coated with 100 µl of matrix proteins in PBS at 37 °C for 1 h. Wells

were washed with PBS and then blocked with 1% BSA in DMEM. Approximately 50,000 CH-8 cells were added to each well in 200 µl of serum-free DMEM containing 0.5% bovine serum albumin. Plates were centrifuged at 10 × *g* for 1 min and then incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed by centrifugation topside down at 48×*g* for 5 min. The attached cells were fixed with 1% formaldehyde and stained with 0.5% crystal violet, and the excess dye was washed off with PBS. The cells were solubilized in 50 µl of 2% Triton-X-100 and quantified by measuring the absorbance at 595 nm in a microplate reader (TECAN, Maennedorf, Switzerland). In each experiment, control wells were coated with BSA, and the absorbance values for these wells were subtracted from those of the test wells. The absorbance of the control wells was not greater than 0.08 throughout the experiments.

Western blotting-CH-8 cells were serum-starved overnight. Cells were then put in suspension on a BSA-coated dish for 4 hrs to reduce basal signals. These cells were then plated in a well of a 6-well plate coated with GTP-free or GTP-bound TG2 for 0 or 30 minutes. The cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X) including a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and the Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). Aliquots of 0.01 mg protein from each sample were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Antibodies against FAK and phosphorylated FAK were used at 1:500 dilutions. Luminescent signals were detected using the ECL reagent (GE Healthcare, Chalfont St. Giles, England) according to the manufacturer's instructions.

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Figure Legends

Figure 1. β1 integrin-dependent hypertrophic differentiation of chondrocytic cells incubated with GTP-bound TG2. Immortalized human chondrocytes (CH-8 cells) were incubated with or without GTP-bound TG2 in the presence or absence of anti-β1-blocking antibody for five days. *Panel A*. Gene expressions of MMP-13, Col X, Col II, and Aggrecan were analyzed by quantitative RT-PCR. *Panel B*. Matrix calcification and ALP activity were analyzed as described in Experimental Procedures. Each bar represents the mean (±S.D.) of triplicate wells.

Figure 2. Expression of β1-integrins on immortalized chondrocytic cell line CH-8. *Panel A.* Expression of α1, α2, α3, α4, α5, α6, and α9 integrin subunits were analyzed by flow cytometry. Open column indicates control staining without primary antibody. *Panel B*. Agarose gel electrophoresis of RT-PCR products of integrins α7, α8, α10, and α11 integrin subunits. *Panel C.* Ct (cycle threshold) values of the quantitative RT-PCR. Ct>40 indicated no expression.

Figure 3. Effects of blocking antibodies against individual β1 integrins on GTP-bound TG2-induced hypertrophic differentiation of chondrocytic cells. *Panel A*. Immortalized human chondrocyte CH-8 cells were incubated with soluble GTP-bound TG2 in the presence of antibodies against α1, α2, α3, α4, α5, or α6 integrin subunits (right gray) or in the presence of a combination of antibodies against α3, α4, and α5 integrin subunits (dark gray and black). The dotted line indicates baseline-values, which correspond to those from cells incubated without TG2. Antibodies are indicated beneath each bar. Each bar represents the mean (± S.D.) of triplicate wells.

Figure 4. Hypertrophic differentiation of chondrocytic cells on immobilized TG2 with or without GTP-bound and inhibition with anti-α5 antibody. Immortalized human chondrocyte CH-8 cells were incubated in wells coated with GTP-bound or free TG2 in the presence or absence of an anti-α5 blocking antibody for five days. MMP-13 and Col X gene expression was analyzed by quantitative RT-PCR. Matrix calcification and ALP activity were analyzed as in Experimental Procedures. The dotted lines indicate baseline values, which correspond to values of cells incubated without TG2. Each bar represents the mean (± S.D.) of triplicate wells.

Figure 5. Adhesion of chondrocytic cells to immobilized GTP-bound TG2. Immortalized human chondrocyte CH-8 cells were plated in wells coated with GTP-bound TG2 at a concentration of 10 μg/ml in the presence or absence of antibody against α3 (P1F6), α4β1 (P3D10), α5β1 (JBS5), or β1 (P5D2). Antibodies used are indicated under each bar. Cell adhesion is expressed as absorbance at 595 nm. Mean value of adhesion to BSA-coated control well was subtracted. Each bar represents the mean (±S.D.) of triplicate wells.

Figure 6. Phosphorylation of Focal Adhesion Kinase (FAK) of chondrocytic cells and inhibition with anti-α5 antibody. CH-8 cells were plated in dishes coated with either BSA (*lane 1*) or 10 μg/ml GTP-bound TG2 (*lanes 2-4*) for 10 (*lane 2*) or 30 min (*lane 3*) in the presence (*lane 4)* or absence (*lane 2,3*) of the blocking anti-α5 integrin subunit monoclonal antibody JBS5. Adherent and non-adherent cells were lysed, and an equal amount of protein from each lysate was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with an antibody to phosphorylated FAK (pFAK). The blots were stripped and re-probed with an antibody to FAK to ensure equal amounts of input protein.

Figure 7. Inductive effect of integrin α5β1 clustering on hypertrophic differentiation of chondrocytic cells. CH 8 cells were plated in wells coated with anti-α5β1 (JBS5; 1, 10, 20 and 50 μg/ml as indicated beneath each bar) for five days. Open bars indicate values of control wells without coating. Gene expression of MMP-13 and Col X were analyzed by quantitative RT-PCR. Matrix calcification and ALP activity were analyzed as described in Experimental Procedures. Each bar represents the mean (± S.D.) of triplicate wells.

Figure 8. Adhesion of chondrocytic cells to immobilized GTP-free or GTP-bound TG2 and the effects of anti-α5 antibody. *Panel A.* CH-8 cells were plated in wells coated with GTP-free or GTP-bound TG2 at a concentration of 10 μg/ml in the presence or absence of an antibody against the α5 integrin subunit (JBS5). *Panel B.* Dose-dependent adhesion of CH-8 cells to GTP-free and GTP-bound TG2 at concentrations ranging from 0-30 μg/ml. Cell adhesion is expressed as absorbance at 595 nm. The mean value of adhesion to BSA-coated control well was subtracted from each experimental value. Each bar represents the mean (± S.D.) of triplicate wells.

Figure 9. Effects of soluble GTP added to cultures of CH-8 cells plated on GTP-free TG2 and on α5β1 antibody. *Panel A.* Soluble GTP (2.5, 25, 250, and 2500 μM as indicated beneath each bar) was added to CH-8 cell cultures plated on GTP-free TG2. *Panel B.* A final concentration of 2.5 μM soluble GTP was added to cultures of CH-8 cell plated on 10 μg/ml immobilized anti-α5β1 antibody. The dotted lines indicate baseline values, which correspond to values when cells were incubated without TG2 or antibody. Each bar represents the mean (±S.D.) of triplicate wells

Figure1

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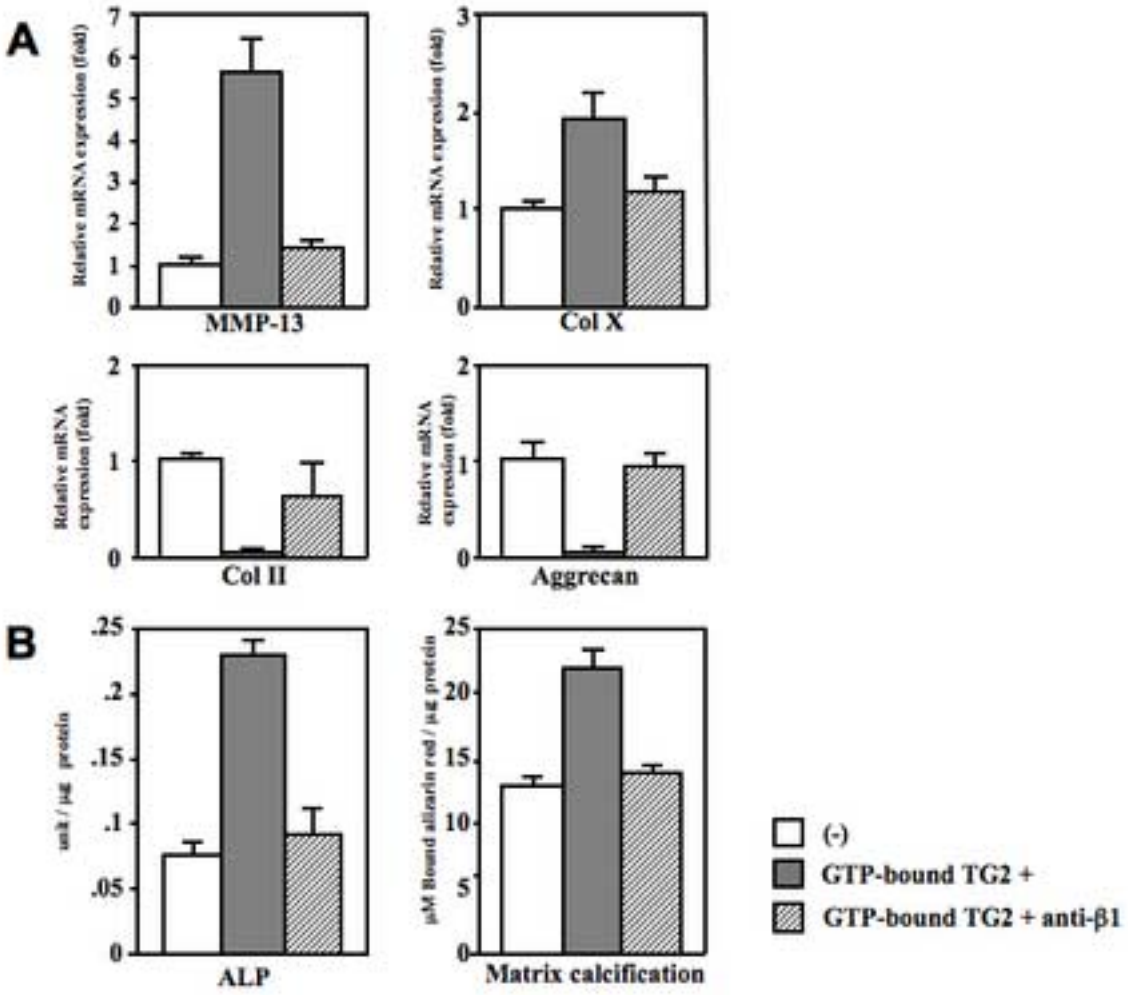
Fig.1



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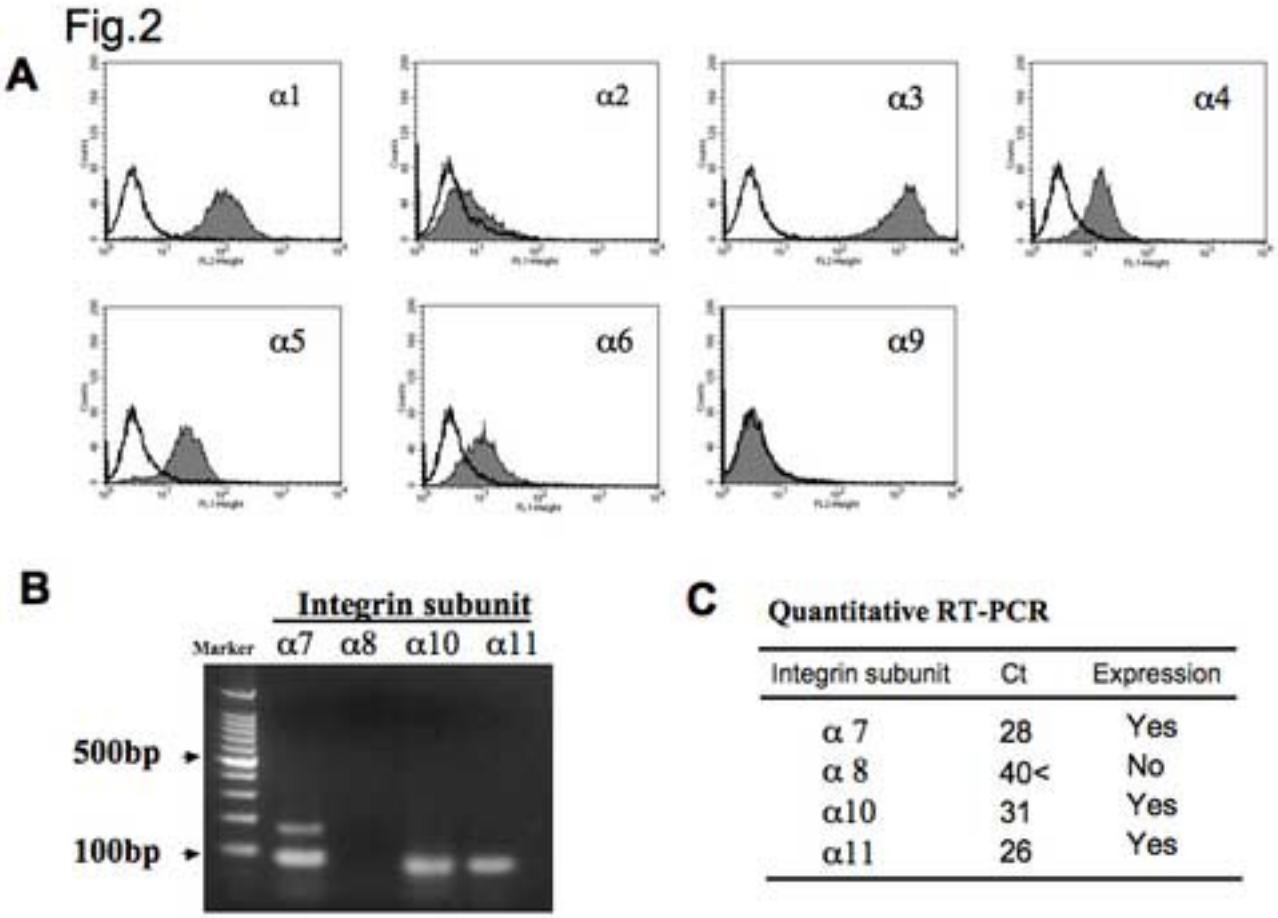

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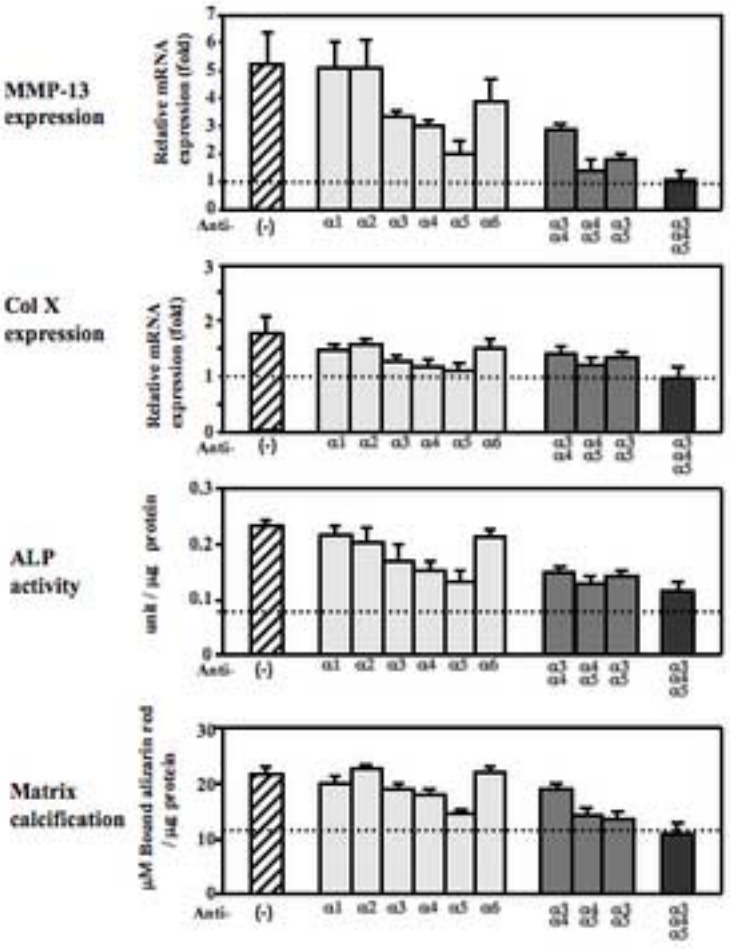

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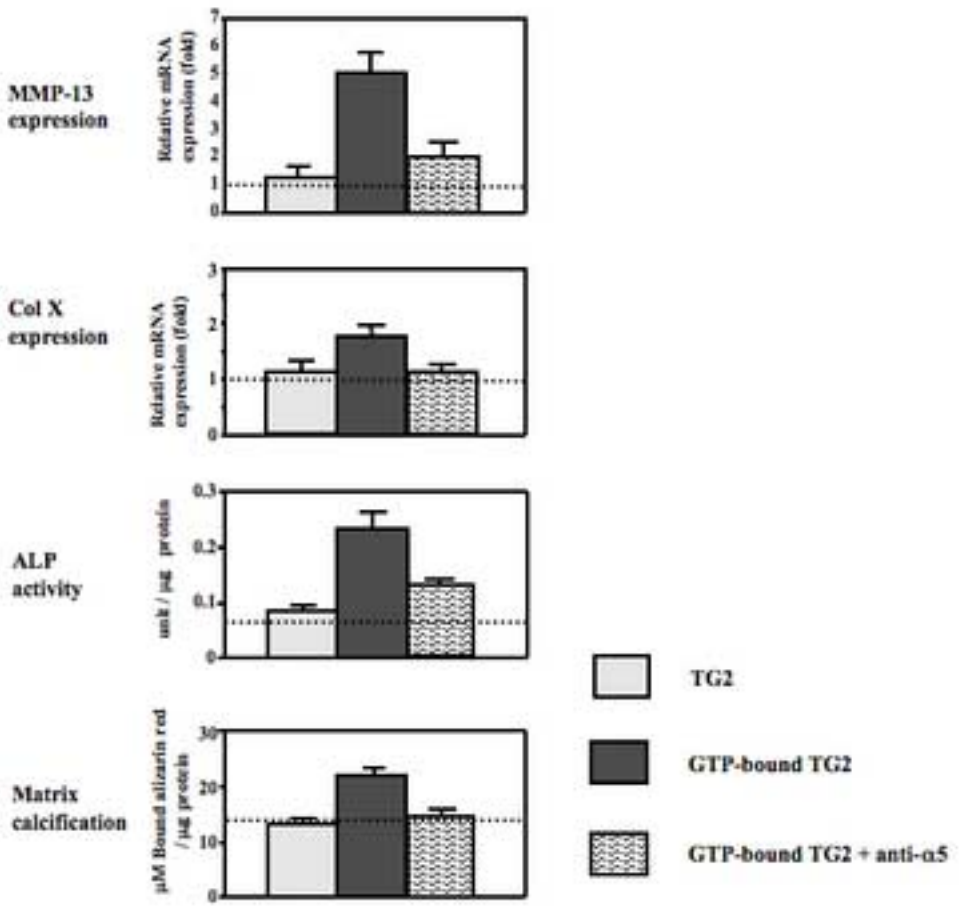

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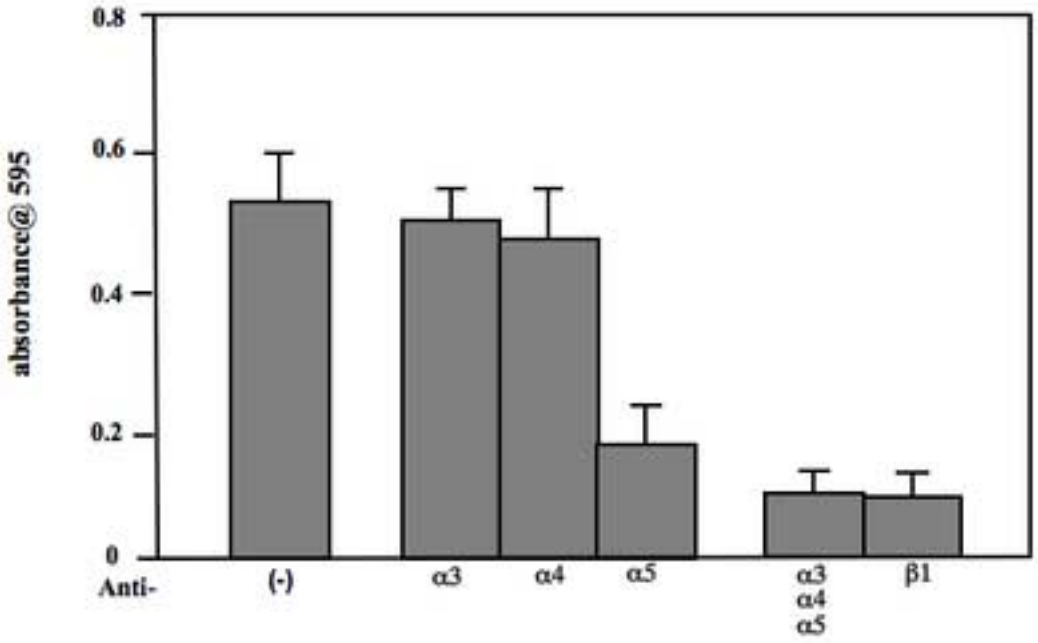

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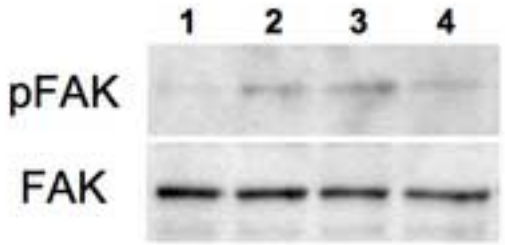

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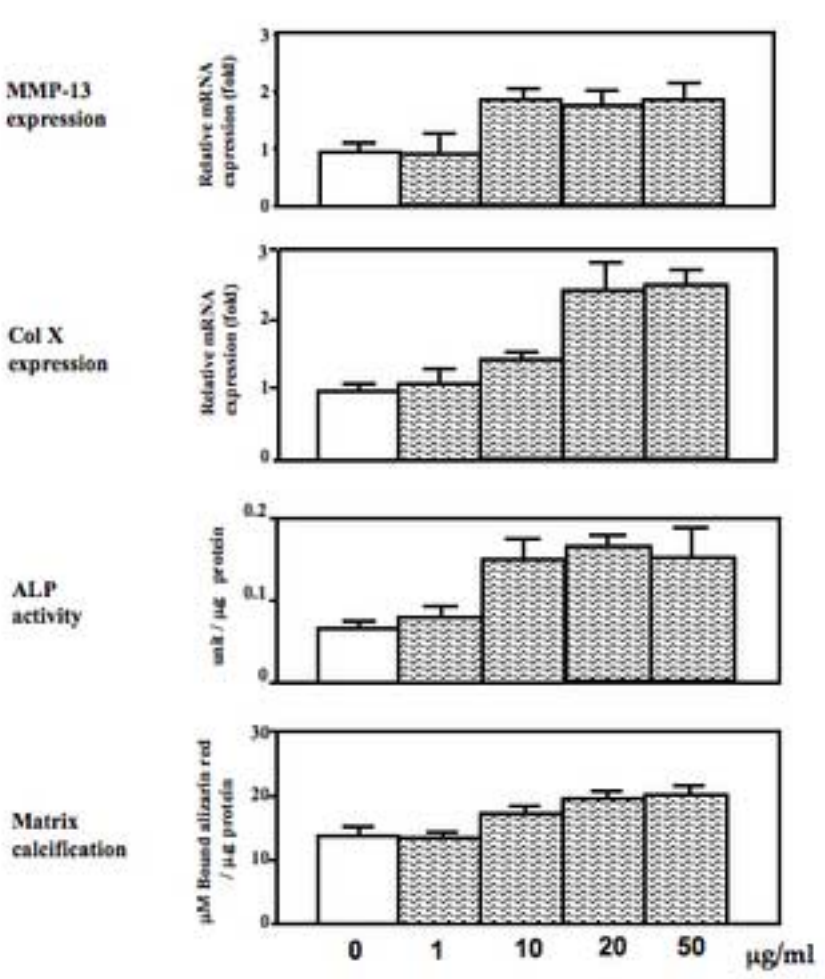

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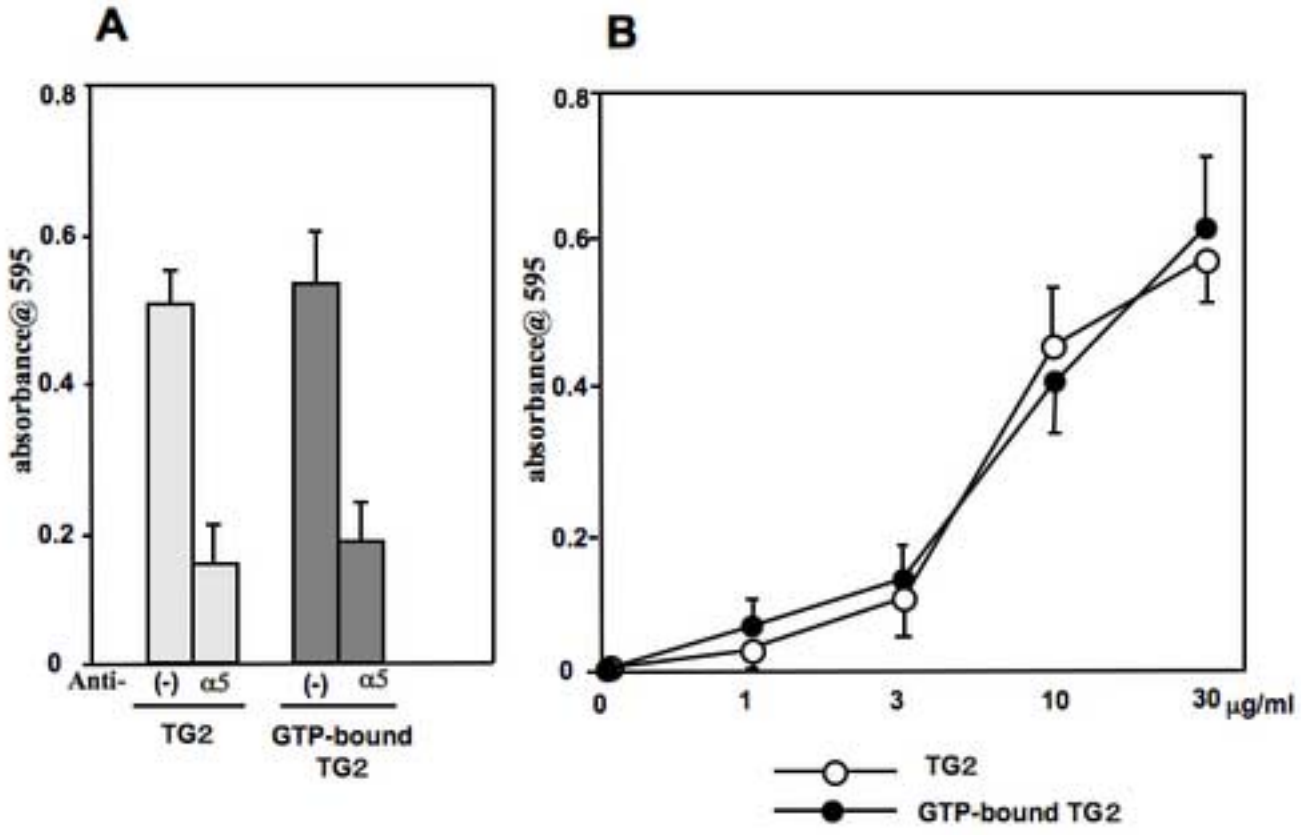

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