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Transforming JB6 Cells Exhibit Enhanced Integrin-Mediated Adhesion to Osteopontin

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Abstract Transformation of preneoplastic epidermal JB6 cells with tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is an in vitro model of late-stage tumor promotion. Osteopontin (OPN) is a secreted, adhesive protein that is highly expressed in JB6 cells with TPA treatment, and its expression persists for at least 4 days, which is the time required for subsequent expression of transformed phenotype. These observations suggest that OPN may play a role in promoting JB6 cell transformation. To function in transformation of JB6 cells, OPN must bind to the surface of the JB6 cell and subsequently signal within the cell. Therefore, we investigated whether JB6 cells adhere to OPN and, if so, to which surface receptors. TPA-treated JB6 cells had significantly ($P < 0.05$) increased adherence to OPN compared with dimethylsulfoxide-treated control cells. Enhanced attachment of JB6 cells to OPN was also observed after treatment with another tumor promoter phorbol dibutyrate but not with nontumor promoters (phorbol and 1α,25-dihydroxyvitamin D_3), suggesting that tumor promoters specifically modulate attachment to OPN. The argininylglycylaspartic acid (RGD) cell-binding region of OPN mediates attachment of TPA-treated JB6 cells because RGD, but not argininylglycylglutamic acid (RGE), peptides inhibited adherence of these cells to OPN in a dose-dependent manner. Flow cytometric analyses, blocking adhesion assay using anti-α_v antibody, and co-immunoprecipitation assay all indicated that TPA-treated cells had similar levels of α_v and β_5 but decreased levels of β_1 compared with untreated cells and that cell adhesion to OPN is most likely mediated through the $\alpha_v\beta_5$. Furthermore, calphostin C, a specific protein kinase C (PKC) inhibitor, decreased TPA-treated JB6 cell adhesion to OPN by 50%, suggesting that TPA increased integrin affinity or avidity for OPN through a PKC-mediated pathway. Collectively, these results indicate that transforming JB6 cells adhere to OPN through its RGD sequence. The most likely OPN receptor is the $\alpha_v\beta_5$ integrin, which increases the affinity or avidity for OPN through a PKC-dependent pathway rather than increasing the number of receptors. J. Cell. Biochem. 78:8–23, 2000.

Key words: osteopontin; integrin; cell attachment; PMA; mouse epidermal cells; tumorigenesis; anchorage-independent growth assay

The extracellular matrix protein, osteopontin (OPN), is a highly acidic glycoprotein found in several tissues and body fluids. In addition to its expression in normal tissues, increased synthesis is associated with pathologic tissues such as transformed or tumorigenic cells [Butler, 1991; Denhardt and Guo, 1993; Patarca et al., 1993; Giachelli et al., 1997; Oates et al., 1997; Rittling and Nowak, 1997; Rittling and Denhardt, 1999]. Comparison of OPN amino acid sequences among various species indicated at least two conserved functional features. The first feature is the consecutive sequence of 7–10 aspartic acids located at the N-terminus of OPN postulated to bind calcium or hydroxyapatite [Oldberg et al., 1986; Prince, 1989]. The second feature is the cell-binding argininylglycylaspartic acid (RGD) sequence shown to functionally adhere to cell surface receptors [Chambers et al., 1993; Loeser, 1993; Ross et al., 1993; Grano et al., 1994; D'Errico et al., 1995; Liaw et al., 1995; Andersson and Johansson, 1996].

The known cell surface receptors for the RGD cell-binding sequence of OPN are the in-

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tegrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ [Reinholt et al., 1990; Miyauchi et al., 1991; Felding-Habermann et al., 1992; Grano et al., 1994; Giachelli et al., 1995; Liaw et al., 1995; Hu et al., 1995]. In addition to this RGD cell-binding sequence, the C- and N-terminal regions of OPN may function in cell adhesion [van Dijk et al., 1993; Nasu et al., 1995; Katagiri et al., 1996; Smith et al., 1996]. The cell surface receptors reported to mediate the adhesion to the non-RGD regions are the hyaluronic acid receptor CD44 [Weber et al., 1996; Katagiri et al., 1999] and the integrins $\alpha_4\beta_1$ [Nasu et al., 1995; Bayless et al., 1998], $\alpha_5\beta_1$ [Nasu et al., 1995], and $\alpha_9\beta_1$ [Smith et al., 1996].

These two functional characteristics of OPN, the ability to bind to calcium and to cell surface receptors, have led to the postulation of functional roles for OPN in tumorigenesis and metastasis [Denhardt and Guo, 1993; Oates et al., 1997]. The association of OPN to tumorigenesis was observed in the early 1980s, before OPN was cloned from an osteosarcoma cell line [Oldberg et al., 1986] and purified from rat bone [Prince et al., 1987]. This protein was initially called transformation-associated secreted phosphoprotein because, regardless of how cells became transformed, e.g., spontaneously, chemically, or by oncogenic viruses, they secreted high levels of the phosphorylated protein, which was later identified as OPN [Senger et al., 1983; Senger et al., 1989].

A second line of evidence linking OPN to tumorigenesis was independently discovered using the promotable preneoplastic mouse JB6 epidermal cell line as a model for late-stage tumor promotion [Colburn et al., 1978, 1979]. Treatment of these cells with the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), under conditions that induce transformation [Colburn et al., 1979] greatly enhanced the expression of an mRNA, which was called 2ar. Sequencing of 2ar cDNA led to its identification as mouse OPN. Because TPA irreversibly transforms JB6 promotable clonal cells, OPN induction by TPA was suggested to be associated with transformation of JB6 cells [Smith and Denhardt, 1987; Craig et al., 1989]. Later in vivo studies further supported the positive association of OPN to tumorigenesis, at least to maintenance of tumor phenotype. Murine epidermal squamous cell carcinomas initiated and promoted by 7,12-dimethylbenz[a]anthracene and TPA, respectively, showed enhanced OPN mRNA expression [Craig et al., 1990].

Although numerous studies have suggested an association of OPN with tumorigenesis, its role in tumor promotion is not clear. An excellent model for studying late-stage tumor promotion is the clonal preneoplastic mouse epidermal cell line, JB6 Cl41.5a (JB6). Treatment of these cells with the tumor promoter TPA results in profound morphologic changes, mitogenic stimulation, anchorage-independent growth (in vitro assay of tumorigenic transformation), and tumorigenesis in nude mice, all hallmarks of transformation to tumor cell phenotype [Colburn et al., 1979; Takahashi et al., 1986; Colburn et al., 1988; Chang and Prince, 1991] and of increased production and secretion of OPN [Smith and Denhardt, 1987; Craig et al., 1989; Chang and Prince, 1991]. OPN mRNA is expressed in JB6 cells as early as 2–4 h after TPA treatment and persists for 4 days [Smith and Denhardt, 1987] (Tucker et al., unpublished data), which is the time required to determine the subsequent expression of transformed phenotype in JB6 cells [Dion et al., 1988]. Our preliminary data suggest that OPN itself is a co-promoter for tumorigenic transformation of JB6 cells (manuscript in preparation), thus implying that the secreted OPN can act in an autocrine manner by interacting either directly or indirectly with the cell surfaces of JB6 cells. Furthermore, OPN induction may be a required event in tumor promotion, as at least two classes of transformation inhibitors, namely retinoic acid and glucocorticoids, also inhibit OPN induction in TPA-treated JB6 cells [Smith and Denhardt, 1989; Dong et al., 1994] (Chang, unpublished data).

In the present study, we determined whether JB6 cells treated with or without TPA could adhere to OPN and, if so, what receptors might be involved. We found that tumor promoter–treated JB6 cells have markedly greater adherence to OPN than do control cells and that the adhesion is mediated by interaction of the RGD cell-binding sequence in OPN and most likely the activated integrin $\alpha_v\beta_5$ expressed on the cell surface.

MATERIALS AND METHODS

Cell Culture

Preneoplastic mouse epidermal cells, JB6 Cl41.5a (generously provided by Dr. N.H. Col-

burn, National Cancer Institute, Frederick, MD) were grown in Minimum Essential Eagle's Medium (MEM) supplemented with 5% heat inactivated fetal bovine serum (FBS), 2% glutamine (GLN), and 0.5% antibiotics. Cells were subcultured before reaching confluent state and checked routinely for *Mycoplasma* contamination by DNA fluorochrome staining [McGarrity et al., 1983]. Cell numbers were determined using a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL).

Extracellular Matrix Proteins

Human fibronectin (FN) was purchased from Boehringer Mannheim (Indianapolis, IN). Vitronectin (VN) was a generous gift from Dr. Candece Gladson (University of Alabama at Birmingham). Fatty acid–free bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). Glutathione S-transferase (GST) and recombinant protein, GST fused to mouse OPN (GST-mOPN), and recombinant mutant forms (deleted RGD-mOPN and RGE-mOPN) were synthesized as described previously [Xuan et al., 1995].

Antibodies

Antibodies to integrins or integrin subunits were used in blocking adhesion assays or flow cytometric analyses. Blocking antibodies recognizing mouse α_v and α_5 were monoclonal antibodies (mAbs) H9.2B8 and 5H10-27, respectively (PharMingen, San Diego, CA). Nonblocking mAbs to mouse β_1, β_3, and human β_5 were 9EG7, 2C9.G2 (PharMingen) and B5-IVF2 (Upstate Biotechnology, Lake Placid, NY), respectively. The rat anti-mouse CD44 mAb was purchased from Chemicon International Inc. (Temecula, CA). The corresponding hamster, mouse, and rat IgG monoclonal immunoglobulin isotype standards (PharMingen) were used in both blocking adhesion assays and flow cytometric analyses. For immunoprecipitation analysis, rabbit anti-human α_v antibody (Ab; Chemicon International Inc.), rat anti-mouse β_1 mAb (9EG7), and rabbit anti-human β_5 Ab (generous gift from Dr. M. Hemler, Dana-Farber Cancer Institute, Boston, MA) were used.

Protein Coating of 96-Well Plates

Matrix proteins were diluted to a final concentration of 10 μg/ml in phosphate buffered saline (PBS), unless specified otherwise. Ninety-six–well plates were coated with 100 μl of each protein or with negative controls (1% BSA or 5 μg/ml of GST) in quadruplicate wells. The number of moles in 100 μl of 5 μg/ml GST is equivalent to the number of moles in 100 μl of GST-mOPN, GST-hOPN, deleted RGD-mOPN, or RGE-mOPN at 10 μg/ml. By enzyme-linked immunoadsorption assay, we have shown that the coating efficiencies of FN and various forms of GST-mOPN to 96-well plates were comparable. Wells were coated with proteins overnight at 4°C and then rinsed with PBS. The plates were blocked with 1% BSA for 2 h at 37°C and rinsed with PBS before cell adhesion assay, as described below.

Cell Adhesion Assays

Before adhesion assay, JB6 cells were treated for 24 h with vehicle 0.001% dimethylsulfoxide (DMSO) or drugs such as 16.3 nM TPA, phorbol dibutyrate (PDBu), or phorbol (Calbiochem, San Diego, CA), or 24 nM 1α,25-dihydroxyvitamin D_3 (calcitriol), generously provided by Dr. L. Binderup (Leo Pharmaceutical Products, Denmark). Cells were harvested using 0.05% trypsin and ethylene-diaminetetraacetic acid (EDTA) and resuspended in MEM containing 0.5% BSA, 2% GLN, and 0.5% antibiotics. A final concentration of 20 μg/ml cycloheximide was added to prevent synthesis of secreted proteins that might have cell adhesive properties [Zerlauth and Wolf, 1985; Chang and Prince, 1993]. Cells (2.5×10^4 cells/100 μl) were transferred to 96-well plates precoated with extracellular matrix proteins and blocked with 1% BSA before initiating the adhesion assay. As a positive control, quadruplicate uncoated wells were plated with control (0.001% DMSO) or TPA-treated cells suspended in MEM containing 5% heat-inactivated FBS, which contained two major cell adhesive matrix proteins, VN and FN [Hayman et al., 1985]. After 90 min of incubation at 37°C in 95% air/5% CO_2, unattached cells were rinsed off with PBS. The number of attached cells was assessed by trypsinizing the cells and counting them by using a ZM Coulter Counter or by hexosaminidase assay, described below.

In blocking adhesion assays, antibodies were incubated with cycloheximide-treated cells in suspension for 30 min before plating onto 96-well plates. Cells were incubated with protein-

coated wells for only 20–30 min instead of 90 min because a longer incubation period led to eventual cell adhesion due to rapid internalization of the integrin and antibody complex through the process of endocytosis [Raub and Kuentzel, 1989; Gaietta et al., 1994].

Hexosaminidase Assay

Endogenous hexosaminidase activity was determined by adding substrate (3.75 mM *p*-nitrophenol-N-acetyl-β-D-glucosaminide, 0.25% Triton X-100 in 0.05 M citrate buffer) to cells adhering to 96-well plates for 2.5 h at 37°C. The enzymatic reaction was terminated by addition of 5 mM EDTA in 50 mM glycine buffer, pH 10.4, and the absorbance was measured using a Titertek Multiscan (Molecular Devices Corporation, Palo Alto, CA) at 405 nm. This enzymatic assay correlates very well with the number of cells attached to 96-well plates [Landegren, 1984].

Flow Cytometric Analysis of Cell Surface Receptors

The clonal JB6 Cl41.5a cells in 150- × 25-mm dishes were pretreated for 24 h with TPA or DMSO before harvesting. Cells were resuspended in 1% BSA in PBS at pH 7.4 and counted using a Coulter counter. Cells from each treatment were divided into 5×10^5 cells/tube and incubated with integrin subunit antibodies or matched isotype monoclonal immunoglobulin standards for 45 min. Cells were rinsed before incubating with secondary antibodies conjugated to fluorescein isothiocyanate for 30 min. These cells were again washed and then fixed using 1% paraformaldehyde at pH 7.4. Stained cells were assayed by flow cytometry using FACSCalibur from Becton Dickenson (San Jose, CA).

Immunoprecipitation of Integrin Subunits

Cells treated with TPA for 24 h were labeled with [^{35}S]methionine/cysteine (0.2 mCi/ml) in methionine and cysteine-free medium for 3 h before the end of the treatment. Cells were rinsed with PBS and lysed in extraction buffer (100 mM NaCl, 1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% NaN_3 in 20 mM Tris, pH 7.4, 2 mM phenyl methyl sulfonyl fluoride, 10 mM ϵ-amino-*n*-caproic acid, 10 mM benzamidine-HCL). Extracts were centrifuged for 30 min at 10,000*g*, and supernatants were incubated with 100 μl of preimmune serum conjugated to 20% protein-A or -G sepharose. After 2 h of incubation, cell lysates were centrifuged, and supernatants were immunoprecipitated for another 2 h at 4°C with purified rabbit anti-human α_v Ab, rat anti-mouse β_1 mAb, or rabbit anti-human β_5 Ab. The antibody–integrin complexes were further incubated overnight with 100 μl of 20% protein-A or -G sepharose. The pellets (preimmune and antibody samples) were washed with extraction buffer, dissolved in nonreducing sample buffer, and subjected to 5% sodium dodecylsulfate–polyacrylamide gel electrophoresis. The gels were fixed, treated for fluorography, dried, and exposed to X-ray film. The immunoprecipitated and co-immunoprecipitated bands were scanned using Umax SuperVista S12, and their intensities were determined using Adobe Photoshop 4.0.

Statistical Analysis

Data are expressed as means ± standard deviations. Statistical analyses were performed by one-way analysis of variance for multiple group comparisons (Figs. 2, 3, 4B and 6). Paired *t* test was performed for the data shown in Figures 4A and 5.

RESULTS

The Number of JB6 Cells Correlates with Hexosaminidase Activity

The number of attached cells in adhesion assays is usually determined by releasing the attached cells by treatment with a protease and a Ca^{2+} chelator (such as trypsin-EDTA) and then enumerating the number of released cells in a hemocytometer or Coulter Counter. However, this method of releasing the attached cells is variable and slow and can damage the cells. Hexosaminidase is an endogenous lysozomal enzyme that is constitutively expressed, and its activity correlates with cell number for other human, rat, and mouse cells [Landegren, 1984]. To determine whether hexosaminidase activity in JB6 cells is directly proportional to the number of JB6 cells, we compared the hexosaminidase activity of attached JB6 cells to parallel wells in which the adherent cells were released by trypsin-EDTA and enumerated on a Coulter Counter. For the cell numbers evaluated (between 2,000 and 25,000), the hexosaminidase activity is directly proportional to the number of JB6 cells, with a correlation coefficient of 0.95 (Fig. 1). TPA does not appear

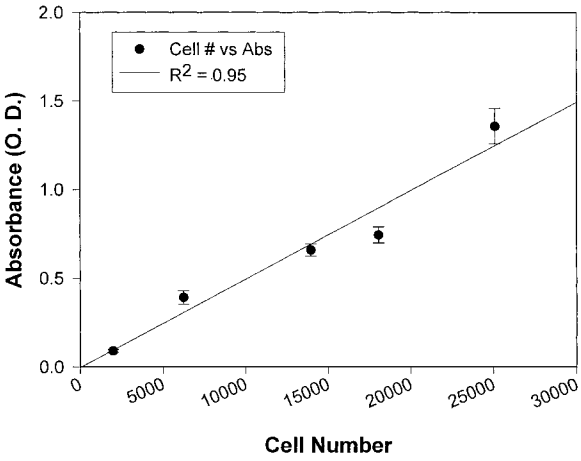

Fig. 1. Correlation of hexosaminidase activity to cell number. Increasing numbers of cells (2,000, 6,000, 14,000, 18,000, and 25,000 cells) were seeded in quadruplicate wells of 96-well plates containing 5% FBS, 2% GLN, and 0.5% antibiotics in MEM. Cells were incubated for 90 min and assayed for hexosaminidase activity, as described in Materials and Methods. Parallel wells were also seeded with the same number of cells and used for direct cell counting by using a Coulter Counter. All values plotted (including those in the figures below) are corrected for the values of blank wells that contained hexosaminidase substrate and stop solution only. Abs, antibodies; O.D., optical density.

to affect hexosaminidase activity because TPA-treated JB6 cells had a similar hexosaminidase activity as untreated control cells. The hexosaminidase activity of JB6 cells reported in optical density (O.D.) units, therefore, was used in all subsequent experiments to assess the number of adherent cells.

TPA Treatment of JB6 Cells Increases Adherence to OPN

To determine whether treatment of preneoplastic JB6 cells for 1 day with the tumor promoter TPA alters their adherence to OPN, we compared the number of TPA-treated JB6 cells in OPN-coated wells with the number of DMSO-treated (vehicle control) JB6 cells. After 24 h of treatment with TPA, the JB6 cells have begun transformation but do not acquire full tumor-like potential until at least 4 days of TPA treatment [Dion et al., 1988]. The cells were treated with cycloheximide before plating in coated wells to prevent the synthesis and secretion of proteins, including endogenous extracellular matrix proteins with cell-binding potential such as OPN and FN [Zerlauth and Wolf, 1985; Chang and Prince, 1993]. The number of TPA-treated JB6 cells bound to GST-mOPN increased in a dose-dependent manner with the concentration of GST-mOPN. For the two highest doses of GST-mOPN (5 and 10 μg/ml), the number of adherent TPA-treated JB6 cells was increased by 28- and 26-fold, respectively, compared with control cells (mOPN-5 and mOPN-10 in Fig. 2A). Neither the TPA-treated nor the control JB6 cells bound to negative control proteins: BSA and GST (BSA and GST in Fig. 2A).

To verify that the vehicle control or DMSO-treated JB6 cells are capable of binding to another extracellular matrix, we plated these cells in FBS-containing medium consisting of VN and FN as the major adhesive proteins in the serum [Hayman et al., 1985]. A large number of control JB6 cells adhered in wells containing FBS (FBS in Fig. 2A), whereas few, if any, control JB6 cells adhered to wells coated with BSA only (BSA in Fig. 2A). However, TPA-treatment of JB6 cells resulted in about 17–50% fewer JB6 cells than control cells adhering to extracellular matrix constituents of FBS (FBS in Figs. 2A,B, 3, 4B, 9).

About 12–20% of preneoplastic TPA-treated JB6 cells acquire transformed characteristics (i.e., the ability to grow anchorage-independently in soft agar). Because JB6 cells require 4 days of TPA treatment for subsequent determination of transformed phenotype in JB6 cells [Dion et al., 1988], we determined whether treatment of these cells with TPA for 4 days retained the enhanced adhesion to OPN as that observed after 1 day of TPA treatment. Indeed, for the highest doses of GST-mOPN (10 μg/ml), the number of adherent TPA-treated JB6 cells was increased by 15-fold compared with the number in control cells (mOPN in Fig. 2B). Thus, the ability of these cells to adhere to OPN even after 4 days of TPA treatment further support the possible role of OPN in enhancing tumor promotion.

In Contrast to Increased Adhesion to OPN, TPA-Treated JB6 Cells Exhibit Decreased Adhesion to FN and VN

FN and VN are extracellular matrix proteins that, like OPN, contain an RGD cell-binding region and are the major cell adhesion proteins of FBS [Hayman et al., 1985]. To determine whether FN and VN, like FBS, results in decreased adherence of TPA-treated JB6 cells, we compared the number of TPA-treated JB6 cells in wells coated with either FN or VN with the

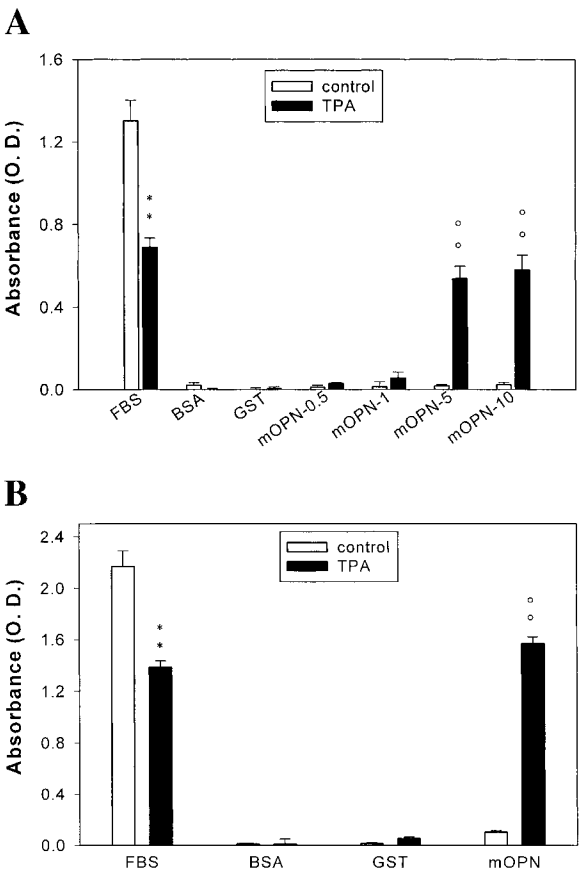

Fig. 2. Enhanced adherence of JB6 cells to GST-mOPN after 1 and 4 days of treatment with TPA. **A:** Cells were treated with DMSO (0.001%) as a control or with TPA (16.3 nM) for 24 h before adhesion assay. Equal cell numbers were added to quadruplicate wells coated with 100 μl of 0.5, 1, 5, and 10 μg/ml (0.05, 0.1, 0.5, and 1 μg, respectively) recombinant GST-mOPN or 5 μg/ml GST (corresponds to equal moles of 100 μl of GST-mOPN at 10 μg/ml) or with 1% BSA (negative control blocking agent) for 90 min. As a positive control, cells were seeded in quadruplicate uncoated wells with MEM containging 5% FBS. The graph represents one of three experiments. Bars represent means ± standard deviations. $^{**}P < 0.01$, adhesion to wells by TPA-treated cells in growth medium is significantly lower than that in control cells (FBS). $^{\circ\circ}P < 0.01$, TPA treatment significantly enhanced cell attachment to OPN at 5 and 10 μg/ml (O.D. values are 0.538 ± 0.059 and 0.580 ± 0.071, respectively) compared with their respective controls (O.D. values are 0.019 ± 0.007 and 0.022 ± 0.010, respectively). **B:** Cells were treated with DMSO as a control or with TPA for 96 h before adhesion assay. The graph is representative of two repeated experiments. $^{**}P < 0.05$, adhesion to wells by TPA-treated cell in growth medium is significantly lower than that in controls (FBS). $^{\circ\circ}P < 0.01$, TPA treatment significantly enhanced cell attachment to OPN at 10 μg/ml compared with control cells.

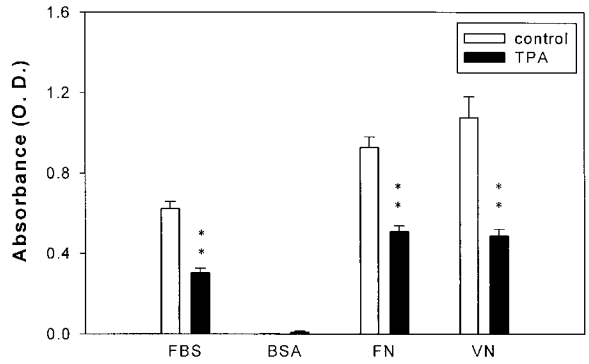

Fig. 3. Decreased adherence of JB6 cells treated with TPA to fibronectin (FN) and vitronectin (VN) compared with control cells. Cells were added to quadruplicate wells coated with 100 μl of 10 μg/ml FN or VN, with BSA as a negative control. The graph represents one of four separate experiments. $^{**}P < 0.01$, the number of TPA-treated cells adhering to FBS and FN- or VN-coated wells is significantly different from that of control cells adhering to respective wells.

number of DMSO-treated JB6 cells in parallel wells. In contrast to the minimal binding to GST-mOPN (mOPN in Fig. 2A,B), control JB6 cells exhibited marked adherence to both FN and VN (FN and VN in Fig. 3). In contrast to the increased binding to GST-mOPN (mOPN in Fig. 2A,B), TPA treatment of JB6 cells resulted in a marked (45% and 55%, respectively) decrease in the number of adhering cells to both FN and VN (FN and VN in Fig. 3). Minimal adherence of either TPA-treated or control cells was observed to a negative control protein BSA (BSA in Fig. 3).

The Tumor-Promoter PDBu Also Induces Marked Adherence of Preneoplastic JB6 Cells to OPN

To determine whether enhanced attachment to OPN by preneoplastic JB6 cells is induced by other phorbol ester tumor promoters, we assessed the number of JB6 cells binding to OPN after incubation with PDBu (a less potent tumor promoter than TPA) or phorbol (which is not a tumor promoter). There was a marked increase in the number of JB6 cells treated with PDBu compared with phorbol-treated cells (mOPN in Fig. 4A); the number of PDBu-treated JB6 cells binding was similar to the number of TPA-treated JB6 cells (mOPN in Fig. 4A). The number of PDBu-treated cells adhering to FBS was similar to the number of TPA-treated cells (FBS in Fig. 4A). Minimal adherence was observed

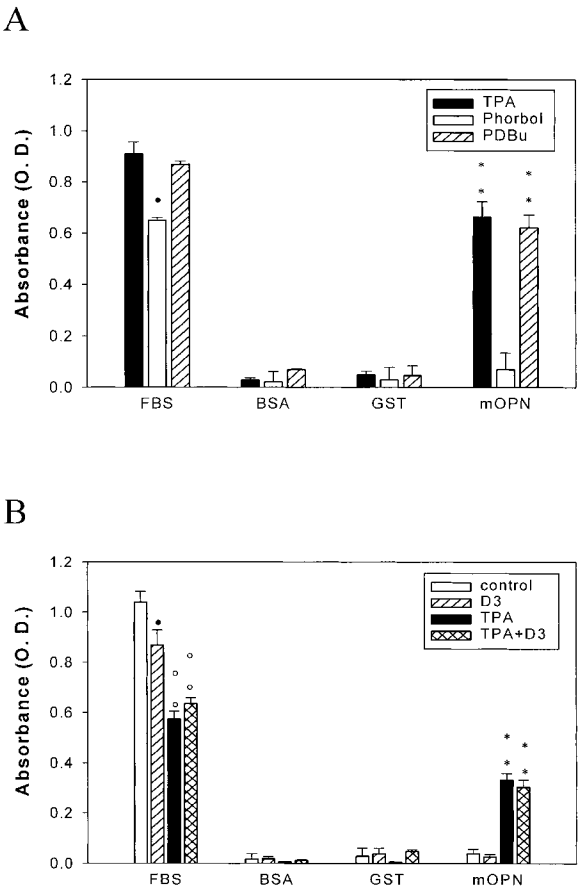

Fig. 4. Adhesion to GST-mOPN of cells treated with phorbol analogs and calcitriol. **A:** Cells were pretreated for 24 h with 16.3 nM of TPA, phorbol dibutyrate (PDBu), or the nontumor promoter phorbol before adhesion assay. Graph represents one of three independent experiments. $^{**}P < 0.01$, adherence to GST-mOPN by TPA- or PDBu-treated cells is significantly greater than that by phorbol-treated cells. $\bullet P < 0.05$, adherence of phorbol-treated cells to FBS is significantly different from that of TPA- and PDBu-treated cells. **B:** Cells were pretreated for 24 h with 2.4×10^{-8} M of calcitriol (D3) and/or 16.3 nM of TPA or DMSO with isopropanol at 0.001% (control) before adhesion assay. Data represent one of two independent experiments. $^{**}P < 0.01$, adherence to GST-mOPN by TPA- or TPA + D3–treated cells is significantly greater than that by control or D3-treated cells. $\bullet P < 0.05$, D3-treated cells adhering to FBS compared with that of control cells is significantly reduced. $^{\circ\circ}P < 0.01$, TPA- and TPA + D3–treated cells significantly show reduced adherence to FBS compared with that of control cells.

to BSA- or GST-coated control wells (BSA and GST in Fig. 4A).

Calcitriol (the active form of vitamin D) acts on TPA-treated JB6 cells to induce further the production of OPN and to enhance further neoplastic transformation of these cells, despite the fact that calcitriol is not considered to be a tumor promoter [Chang and Prince, 1993]. To determine whether this augmentation of neoplastic transformation and OPN production also increases the adherence of these cells to OPN, we performed adhesion assay with TPA-treated JB6 cells incubated for 24 h with calcitriol. Calcitriol-treated JB6 cells (D3 in Fig. 4B) attached minimally to GST-mOPN, whereas a marked number of JB6 cells treated with TPA or with TPA plus calcitriol (TPA + D3 in Fig. 4B) bound to GST-mOPN. Calcitriol, however, did not enhance the adhesion of TPA-treated JB6 cells. As observed in all these experiments, few of the treated cells bound to BSA or to GST and a large number of cells bound to FBS; TPA-treatment of JB6 cells again reduced the number of cells binding to FBS compared with DMSO-treated control cells. The number of TPA- plus calcitriol-treated JB6 cells binding to FBS was similar to the number of TPA-treated cells (FBS in Fig. 4B).

The RGD Cell-Binding Sequence in OPN Mediates the Attachment of TPA-Treated Cells to OPN

To determine the molecular domain within OPN that mediates the attachment of TPA-treated JB6 cells to OPN, we performed cell attachment assays in the presence of RGD or control RGE peptides. Binding of TPA-treated JB6 cells was inhibited (94 ± 8%) by 50 μM RGD peptide but not by the control RGE peptide (<5%), and this inhibition of binding of TPA-treated JB6 cells occurred in a dose-dependent manner (Fig. 5A). The IC_{50} for the RGD peptide was 4 μM.

To confirm that the RGD cell-binding domain of OPN mediates the attachment of TPA-treated JB6 cells to OPN, we performed cell adhesion assays with specifically mutated forms of OPN. Marked numbers of TPA-treated JB6 cells bound to GST-mOPN, but few TPA-treated JB6 cells bound to either OPN in which the RGD cell-binding sequence was deleted or OPN in which the RGD cell-binding sequence was replaced with an RGE sequence (Fig. 5B). Thus, binding of TPA-treated cells was completely inhibited (100%) by mutating the RGD cell-binding sequence.

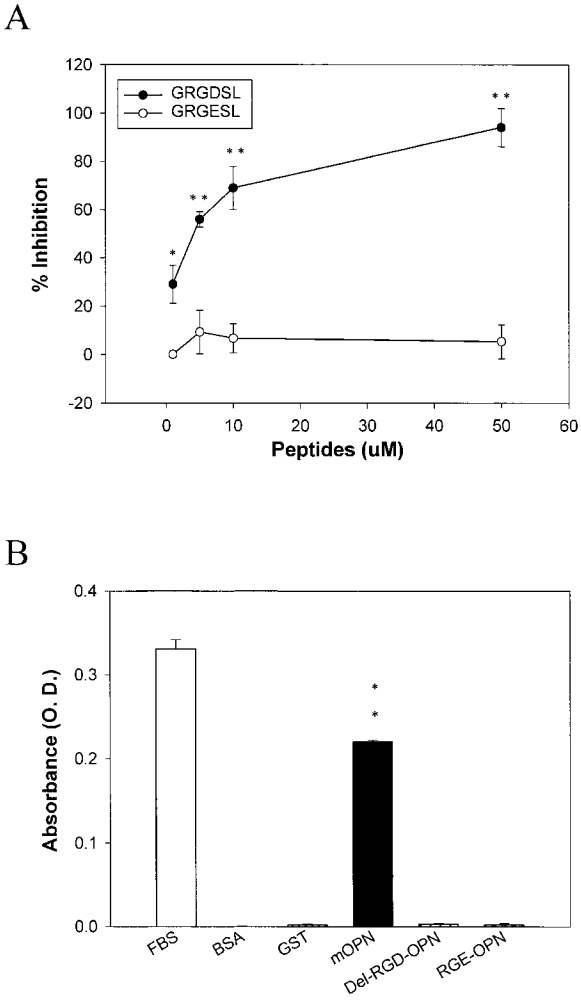

Fig. 5. Competitive binding assay of cells treated with TPA to OPN in the presence of RGD or RGE-containing peptides and comparison of TPA-treated cell adhesion to GST-mOPN versus mutated GST-mOPN (containing deleted RGD or RGD replaced with RGE). **A:** Different concentrations (1, 5, 10, and 50 μM) of GRGDSL or GRGESL peptides were added to TPA-treated cells before transferring to 96-well plates. Results represent the mean ± SE of three experiments performed in quadruplicate for each concentration of peptides. $*P < 05$, $**P < 0.01$, percentage of inhibition of TPA-treated cells adhering to GST-mOPN by GRGDSL peptides is significantly greater than that by GRGESL peptides. **B:** TPA-treated cells were incubated in quadruplicate wells coated with 100 μl of 10 μg/ml GST-mOPN (mOPN), deleted RGD-mOPN (Del-RGD-OPN), or RGE-mOPN (RGE-OPN) or 5 μg/ml of GST. Data represent one of two independent experiments. $**P < 0.01$, adherence of TPA-treated cells to GST-mOPN is significantly greater than to Del-RGD-mOPN or RGE-mOPN.

JB6 Cells Express α_v, α_5, β_1, β_5 Integrin Subunits and CD44 on Their Cell Surface but not the β_3 Integrin Subunit

To assess whether increased adhesion to OPN was due to increased expression of OPN-binding surface receptors by TPA, we compared the cell-surface expression of selected α and β subunits of integrin receptors $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$, previously shown to mediate adhesion to OPN through the RGD-dependent region [Reinholt et al., 1990; Miyauchi et al., 1991; Felding-Habermann et al., 1992; Grano et al., 1994; Giachelli et al., 1995;; Hu et al., 1995 Liaw et al., 1995] on DMSO-treated versus TPA-treated JB6 cells. The level of integrin subunit expression was assessed 24 h after initiation of treatment with DMSO or TPA. Neither control nor TPA-treated JB6 cells expressed β_3 integrin subunit detectable by flow cytometry: the mean fluorescence intensity of control and TPA-treated JB6 cells labeled with anti-mouse β_3 mAb were FI-C: 0 and FI-T: 0, respectively (Fig. 6). To verify the lack of β_3 integrin subunit expression, we performed Northern blot analyses of mRNA from JB6 cells. No β_3 mRNA was detected in either control or TPA-treated JB6 cells (data not shown).

Control JB6 cells did express α_v, β_1, and β_5 integrin subunits with a mean fluorescent intensity (FI-C) of 7, 24 and 6, respectively (Fig. 6). TPA treatment of JB6 cells did not alter the level of cell-surface expression of α_v and β_5 integrin subunits (Fig. 6; FI-T of 8 and 7, respectively) compared with control cells. However, β_1 integrin subunit was reduced by 39% in JB6 cells treated with TPA compared with control cells (Fig. 6; FI-C: 24 and FI-T: 14, respectively; for $n = 3$, β_1 integrin subunit reduction is $30 \pm 0.7\%$, $P < 0.01$). This decreased expression level of β_1 on the surface of JB6 cells persisted for at least 48 h after treatment with TPA (FI-C: 24 and FI-T: 14).

Because the β_1 integrin subunit dimerizes not only with the α_v subunit but also with the α_5 subunit to form the FN receptor ($\alpha_5\beta_1$), the cell surface expression of α_5 was determined in JB6 cells. Control JB6 cells did express α_5, with a FI-C of 61 (Fig. 6). JB6 cells treated with TPA did not have a significant change in cell surface expression of α_5, which has an FI-T of 64 (Fig. 6). These data suggest that enhanced adhesion of TPA-treated JB6 cells to OPN is not likely due to an increase in the integrin receptor

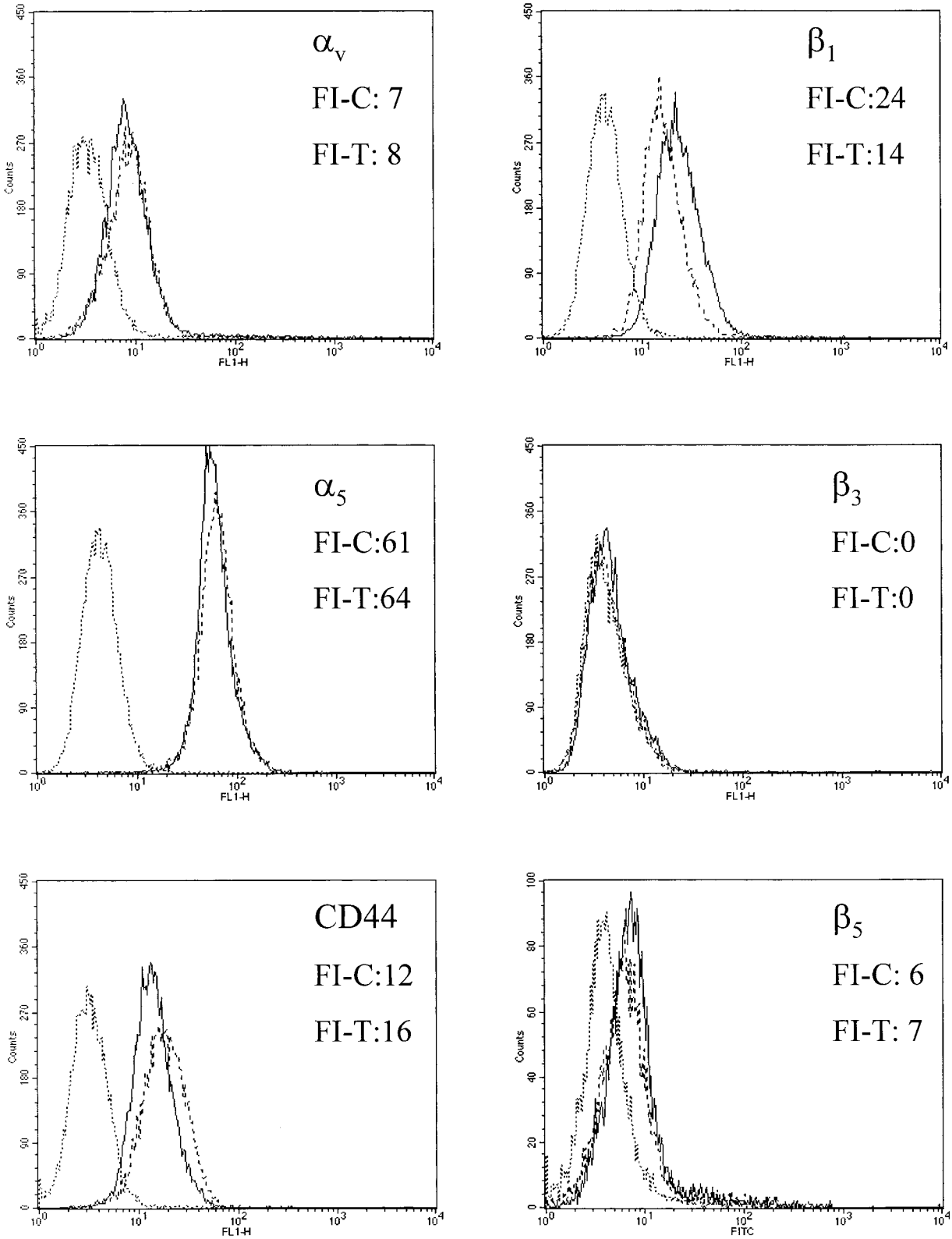
α_v
FI-C: 7
FI-T: 8
β_1
FI-C:24
FI-T:14
α_5
FI-C:61
FI-T:64
β_3
FI-C:0
FI-T:0
CD44
FI-C:12
FI-T:16
β_5
FI-C: 6
FI-T: 7
Counts
FL1-H
FITC

Figure 6.

number because flow cytometric analyses did not indicate any significant increase with the α and β subunits of the relevant integrins ($\alpha_v\beta_1$ and $\alpha_v\beta_5$) in TPA-treated versus control cells; instead, TPA significantly decreased the surface expression of β_1. Thus, we believe that TPA increases the affinity or the avidity of cell surface receptor for OPN.

Because CD44, a hyaluronic acid receptor, found in some tumor cells has been reported to adhere to OPN [Weber et al., 1996], we asked whether TPA-treated versus control JB6 cells express this receptor. CD44 is expressed in both control and 24-h TPA-treated JB6 cells, with FI-C: 12 and FI-T: 16, respectively (Fig. 6). TPA treatment, however, did not significantly increase the surface expression of CD44 even after 48 h of treatment. The FI of CD44 for 48 h of control JB6 cells versus TPA-treated cells were similar at 10 and 9, respectively.

α_v-Containing Integrins Mediate the Adhesion of TPA-Treated JB6 Cells to OPN

To determine whether α_v-containing integrins mediate binding of OPN to TPA-treated JB6 cells, we performed adhesion assays to OPN with TPA-treated JB6 cells that were incubated with blocking anti-α_v mAb before the adhesion assay. Incubation with anti-α_v mAb (OPN-alphaV in Fig. 7) significantly ($P < 0.05$) reduced the number of adherent TPA-treated JB6 cells to GST-mOPN. In contrast, TPA-treated cells either not incubated with mAb (OPN-no Ab in Fig. 7) or incubated with control hamster IgG (OPN-IgG in Fig. 7) bound to GST-mOPN. Repeated experiments indicated that anti-α_v mAb at 50 μg/ml inhibited the adherence of JB6 cells to GST-mOPN by 80 ± 13%, n = 3.

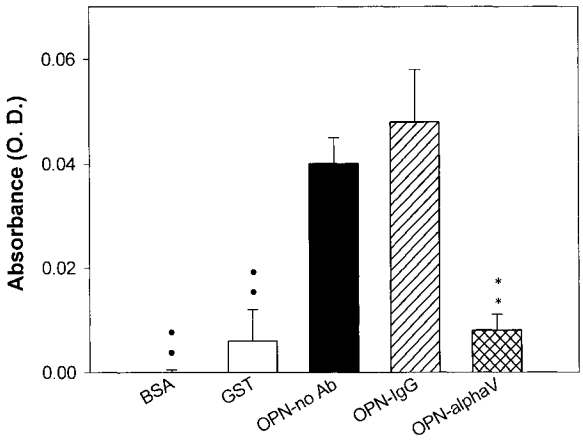

Fig. 7. Inhibition of TPA–treated cell adhesion to GST-mOPN by monoclonal antibody against α_v. Cells were preincubated with antibodies against mouse α_v (OPN-alphaV) or its matched monoclonal isotype antibody, hamster IgG (OPN-IgG), for 30 min before plating on BSA, GST, or GST-mOPN–coated wells for 20 min. Data represent one of three independent experiments. $^{**}P < 0.01$, anti-α_v antibody significantly inhibited the adhesion of TPA-treated cells to attach to GST-mOPN. $\bullet\bullet P < 0.01$, adhesion to BSA and GST by TPA-treated cells is significantly reduced compared with adhesion to GST-mOPN by TPA-treated cells not incubated with α_v antibody (OPN-no Ab) or incubated with hamster IgG (OPN-IgG).

Because the α_v integrin subunit mediates virtually all the binding of TPA-treated JB6 cells to OPN, we wanted to determine which β integrin subunits (β_1 or β_5) are linked to the α_v subunit in these cells. To do this, we performed immunoprecipitation analyses on DMSO- or TPA-treated JB6 cells using the rabbit antibody against α_v. The anti-α_v antibody co-immunoprecipitated bands with molecular weights corresponding to β_1 and β_5 integrin subunits, with the latter being the more predominant band (Fig. 8, lane 4; Table I). Anti-α_v antibody also co-immunoprecipitated an unidentified protein below α_v (Fig. 8) that it is not

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Fig. 6. Comparison of α_v, α_5, β_1, β_3, and β_5 integrin subunits and CD44 expressed on the surface of JB6 cells treated with or without 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Cells were treated with 0.001% of dimethylsulfoxide (DMSO; curve with solid line) or 16.3 nM TPA (curve with wide dotted line) for 24 h followed by staining with monoclonal antibodies (mAbs) against α_v, α_5, β_1, β_3, β_5, and CD44 or with matched monoclonal-specific isotype antibodies (curve with small dotted line). After the addition of secondary antibodies conjugated to fluorescein isothiocyanate (FITC), cells were analyzed by flow cytomety as described in the Materials and Methods. The y axis is representative of cell number, and the x axis is the amount of fluorescence generated by FITC conjugated to secondary antibody bound to either specific mAbs or their control isotype antibodies; 10,000 events were analyzed for each sample. FI (mean fluorescence intensity), mean fluorescence intensity of the integrin subunit antibody minus mean fluorescence intensity of the isotype antibody; FI-C, mean fluorescence intensity of control or DMSO-treated cells; FI-T, mean fluorescence intensity of TPA-treated cells. Data are representative of two or three independent experiments. The scale on the y axis of graph β_5 is one-fourth that of the other graphs because of the change in software from Becton Dickenson used to analyze the data. The β_5 data were analyzed using Cell Quest, and the α_v, α_5, β_1, β_3 and CD44 data were analyzed using Consort 30.

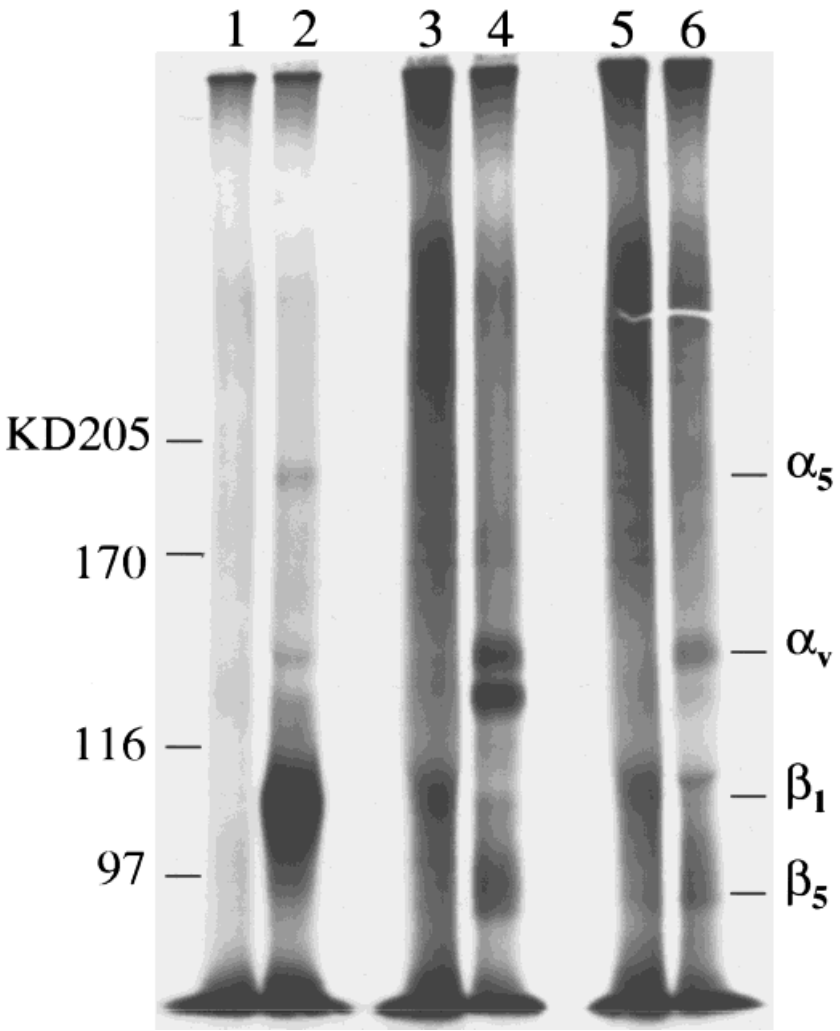

Fig. 8. Sodium dodecylsulfate–polyacrylamide gel electrophoresis fluorogram of radiolabeled glycoproteins, immunoprecipitated with specific antibodies to α_v, β_1, or β_5. The TPA–treated cells were labeled, and immunoprecipitated proteins were separated in 5% SDS gel electrophoresis under nonreducing conditions, as described in Materials and Methods. **Lanes 1, 3, and 5** are proteins precipitated by preimmune rat IgG_{2a}, κ, preimmune rabbit serum, and preimmune rabbit serum, respectively. **Lanes 2, 4, and 6** are proteins precipitated by rat anti-mouse β_1 monoclonal antibody, rabbit anti-human α_v antibody, and rabbit anti-human β_5 antibody, respectively. The integrin β_1 subunit (~110 kDa, lane 2) co-immunoprecipitates with α_5 (~180 kDa) and with α_v (~150 kDa). The integrin α_v subunit (lane 4) co-immunoprecipitates with an unidentified band (~130 kDa), with β_1, and with β_5 (~90 kDa). The integrin β_5 subunit (lane 6) co-immunoprecipitates with α_v.

any of the β subunits (β_1 and β_5) known to be involved in dimerizing with α_v. Furthermore, it cannot be β_3 because in mouse its molecular weight is around 90; also, flow cytometric and Northern blot analyses results did not indicate its presence. The unidentified protein could be a precursor of α_v [Cheresh and Harper, 1987]. To verify our assumption, additional studies are in progress.

The anti-β_1 and -β_5 antibodies both co-immunoprecipitated a band with a molecular weight corresponding to that of the α_v integrin subunit (Fig. 8). However, anti-β_5 antibody co-immunoprecipitated α_v integrin subunit with a 1:1 ratio, whereas anti-β_1 antibody co-immunoprecipitated α_v and α_5 integrin subunits with ratios of 1:0.34 and 1:0.32, respectively (Table I). Taken together, these results suggest that the adhesion of transforming JB6 cells to OPN is more likely mediated through the $\alpha_v\beta_5$ integrin.

Enhanced Attachment of TPA-Treated Cells to OPN Is Mediated Through PKC Activation

We have shown that enhanced binding of JB6 cells to OPN is not due to TPA-induced increased expression of the relevant integrin subunits. To explore the possibility that the enhanced attachment of these cells to OPN is mediated by TPA-induced activation of surface receptor through the protein kinase C (PKC) signaling pathway, a specific PKC inhibitor, calphostin C, was used. Calphostin C is a specific competitive inhibitor of TPA for binding to the regulatory domain of all PKC isoforms. The specific PKC inhibitor at 100 nM decreased the attachment of TPA-treated JB6 cells to OPN by 50% (Fig. 9), suggesting that TPA could acti-

TABLE I. Relative Intensities of Co-Immunoprecipitated Integrin Subunits*

Immunoprecipitating antibody	Intensity of			
	α_v	β_1	β_5	α_5
Anti-α_v Ab	1	0.69*	0.82	—
Anti-β_1 mAb	0.34	1	—	0.32
Anti-β_5 mAb	1.06	—	1	—

*Relative intensities of coimmunoprecipitated α_v, β_1, and β_5 integrin subunits after immunoprecipitation of cell lysate treated with TPA, with the designated antibodies in the left-hand column. The values of each band are normalized to the intensity of the protein being immunoprecipitated because of the potential differences in the efficiency of immunoprecipitation by the different antibodies. For band quantification, see Materials and Methods. Ab, antibody; mAb, monoclonal antibody.

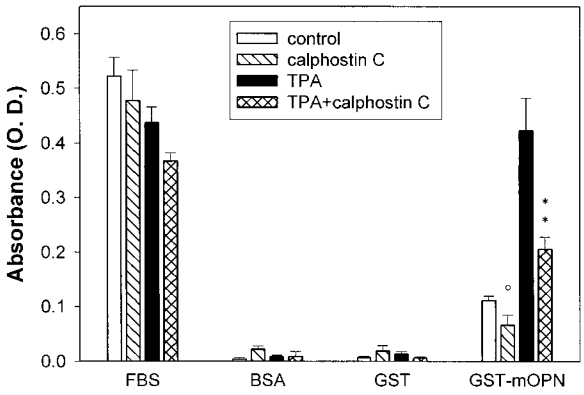

Fig. 9. The protein kinase C inhibitor, calphostin C, diminished the adhesion of TPA–treated cells to GST-mOPN. Cells were treated with 0.008% of DMSO as a control (open bar) or with 100 nM of calphostin C (striped, solide, and cross-hatched bars) for 1 h, followed by the addition of 16.3 nM of TPA (solid bar) for 24 h before adhesion assay. Cells were plated in BSA-, GST-, or GST-mOPN–coated wells. Plot represents one of two independent experiments. $^{**}P < 0.01$, calphostin C at 100 nM significantly reduced TPA-treated cells from adhering to GST-mOPN. $^{\circ}P < 0.05$, calphostin C significantly reduced DMSO-treated cells from adhering to GST-mOPN.

vate directly or indirectly the α_v-dependent integrin and thereby increase its affinity or avidity for OPN.

DISCUSSION

The preneoplastic mouse JB6 model is well characterized for late-stage tumor promotion study [Colburn et al., 1979]. Upon treatment with the tumor promoter TPA, these clonal cells acquire the ability to grow anchorage independently in soft agar and form tumors in nude mice [Takahashi et al., 1986; Colburn et al., 1988]. Osteopontin production and secretion are associated with the transformation of preneoplastic JB6 cells into tumor cells. In addition, retinoic acid and glucocorticoids, known transformation inhibitors, suppress TPA-induced OPN expression in JB6 cells. These observations suggest that secreted OPN may assist JB6 cell transformation, perhaps through an autocrine pathway [Smith and Denhardt, 1987, 1989; Chang and Prince, 1993] (Chang, unpublished data). Therefore, we first investigated whether OPN may interact with the cell surfaces of JB6 cells and, if it does, which region of OPN is involved in mediating binding to which type of integrin surface receptors.

Our observation that transforming JB6 cells adhere markedly to OPN, whereas control JB6 cells have minimal adherence, supports the idea that OPN could play an autocrine role in TPA-treated cells and is consistent with our finding that OPN can act as a co-promoter to transform JB6 cells (Chang et al., manuscript in preparation). While increasing their adherence to OPN, these transforming JB6 cells decrease their adherence to VN and FN, two major constituents of the extracellular matrix, with the latter protein constitutively synthesized by JB6 cells. This decrease in attachment to FN, which is also characteristic of other transformed cell lines, has been shown to result in altered morphology and motility [Yamada, 1983]. Our observation is consistent with that of previous reports that TPA-treated JB6 promotable cell lines have markedly decreased levels of FN associated with their cell surface [Zerlauth and Wolf, 1985], implying that the surface expression of the FN receptor, $\alpha_5\beta_1$ is decreased. Because flow cytometric analyses of the surface integrins on JB6 cells indicate that the β_1 integrin subunit, but not α_5 subunit, in TPA-treated cells is significantly reduced compared with control cells, we postulate that the reduced adhesion of TPA-treated JB6 cells to FN is contributed mainly by the reduction in the β_1 integrin subunit. In addition, we postulate that the morphologic changes initiating as early as 8 h after TPA treatment (data not shown) and persisting for at least 48 h [Fig 7 in Chang and Prince, 1991] may be contributed in part by this observed changes in surface expression of the β_1 integrin subunit. Further studies, however, relating to early time course on β_1 surface expression and

on cell adhesion to FN will be necessary to confirm this notion.

Whereas both control and TPA-treated JB6 cells synthesize FN, VN is not detectable by immunoprecipitation analysis (data not shown). However, VN in FBS is considered the major contributor to cell adhesiveness in culture media [Hayman et al., 1985]. The known receptors that adhere to VN are similar to the OPN receptors, which are RGD mediated: $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. Our observation that transforming JB6 cells have decreased adhesion to VN also suggests that $\alpha_v\beta_1$ may mediate adhesion to VN in these cells because β_1 expression is decreased, β_3 integrin subunit is not present, and β_5 subunit surface expression is not decreased in JB6 cells after TPA treatment. This switch in decreased adherence for VN and FN to increased adherence for OPN, which is synthesized and secreted in large amounts by transforming JB6 cells, suggests the possibility of an autocrine feed-forward loop in which the transforming cells are further stimulated by OPN toward the process of transformation.

This postulation can be further supported by the observations that control, nontransforming JB6 cells, which do not synthesize OPN, have minimal ability to grow anchorage independently in soft agar (an assay system that highly correlates with in vivo tumorigenic transformation) [Colburn et al., 1978], whereas the addition of purified OPN resulted in significant increase in anchorage-independent growth (Chang et al., manuscript in preparation).

The lack of cell transformation in control JB6 cells also implies that VN and FN do not assist in the transformation process of JB6 cells because anchorage-independent growth assay in soft agar normally includes 10% FBS, which contains both cell adhesive proteins VN and FN. In contrast to control cells, treatment with TPA initiates the transformation process, stimulates these cells to synthesize and secrete large amounts of OPN, and, furthermore, enhances cell adhesion to OPN. Consequently, the accumulation of OPN at the surface of transforming cells embedded in soft agar may, by an autocrine loop, further stimulate the transformation process, resulting in colony formation.

The increased binding to OPN appears to be related to the transforming effects of the tumor promoter because TPA and PDBu transform JB6 cells to enable their binding to OPN, whereas phorbol does not. Calcitriol is not normally considered a tumor promoter, but it does markedly enhance the transformation of TPA-treated JB6. Concurrently, calcitriol further enhances OPN production and secretion [Chang and Prince, 1993]. These results of calcitriol treatment of transforming JB6 cells suggested that calcitriol might enhance the process of transformation by increasing adherence to OPN in TPA-treated JB6 cells. However, we found that calcitriol treatment of JB6 cells resulted in minimal adherence to OPN, and calcitriol treatment of transforming JB6 cells did not increase the number of adhering JB6 cells compared with the TPA treatment alone. These data suggest that TPA, but not calcitriol, induces an alteration in the affinity or avidity of the cell surface receptors of JB6 cells for extracellular matrix proteins.

Because JB6 cells adhere to OPN effectively after tumor promoter treatment, we then determined the region of OPN that mediates binding to these cells. The most likely domain on OPN mediating the adherence of transforming JB6 cells is the RGD cell-binding domain because RGD, but not RGE, peptides markedly inhibit the adherence of JB6 cells to OPN. In addition, deletion of the RGD cell-binding region in OPN or replacement of the RGD cell-binding region in OPN with an RGE-containing sequence virtually abrogates adhesion of transforming JB6 cells to these mutated forms of OPN. The absence of binding to the mutated forms of OPN also argues against the other cell-binding domains within OPN contributing to the observed attachment of transforming JB6 cells to OPN.

The cell surface receptor for OPN on transforming JB6 cells is most likely α_v coupled with β_1, β_3, or β_5 integrin subunits because these integrins mediate binding through the RGD region of OPN, and blocking anti-α_v mAb virtually eliminates binding of transforming JB6 cells to OPN. CD44, even though expressed by JB6 cells and shown to mediate binding to OPN in some cells, is unlikely the cell surface receptor for OPN on transforming JB6 cells because CD44 mediates binding to a region of OPN outside the RGD cell-binding sequence [Weber et al., 1996]. The observation that the β_3 integrin subunit is not expressed either at the mRNA or protein level in transforming and control JB6 cells indicates that $\alpha_v\beta_3$ integrin is

not involved in adherence of transforming JB6 cells to OPN.

Thus, there remain two candidate β integrin subunits, β_1 and β_5, that could couple to α_v. Determining which β chain mediates binding is more difficult because there are no blocking antibodies readily available to mouse β_1 and β_5. Our flow cytometric analyses show that the level of expression of the β_1 integrin subunit declines significantly ($P < 0.01$) after JB6 cells begin transforming. Furthermore, the most prominent band in our co-immunoprecipitation analyses using anti-α_v antibody was the β_5, not β_1, integrin subunit. This was also consistent with co-immunoprecipitation analysis using antibodies to β_1 and β_5, where more α_v subunit co-precipitated with β_5 antibody than with the β_1 antibody. These flow cytometric and immunoprecipitation analyses suggest that the $\alpha_v\beta_5$ integrin is more likely to mediate attachment of transforming JB6 cells to OPN. In addition, because TPA treatment did not increase the expression of the relevant integrin subunits involved in adhering to OPN, we postulate that TPA increases the affinity or the avidity of the integrin for OPN and that the mechanism of TPA action is PKC mediated, as supported by the observation that the specific inhibitor of PKC, calphostin C, was able to decrease the adhesion of TPA-treated cells to OPN.

In conclusion, our data suggest that during the transformation process neoplastic JB6 cells acquire an increase adherence for OPN, thereby setting up a potential autocrine loop because transforming JB6 cells also secrete large amounts of OPN. The binding of OPN through its RGD region may signal through the cell surface integrin $\alpha_v\beta_5$ and augment the transformation process in the JB6 cells. Thus, inhibiting this autocrine OPN loop may provide a target for inhibition of tumor development.

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