Tumor Cell Invasion Is Promoted by Activation of Protease Activated Receptor-1 in Cooperation with the $\alpha_v\beta_5$ Integrin*

Received for publication, August 3, 2000, and in revised form, January 25, 2001
Published, JBC Papers in Press, January 26, 2001, DOI 10.1074/jbc.M007027200

Sharona Cohen Even-Ram‡, Miriam Maoz‡, Elisheva Pokroy‡, Reuven Reich§, Ben-Zion Katz‡, Paul Gutwein‡, Peter Altevogt¶, and Rachel Bar-Shavit‡**

From the Departments of Oncology and Pharmacology at the Hadassah-Hebrew University Hospital, Jerusalem 91120, Israel, the Department of Hematology, Medical Center, Tel Aviv 64239, Israel, and the Tumor Immunology Program, German Cancer Research Center, D-69120 Heidelberg, Germany

The first prototype of the protease activated receptor (PAR) family, the thrombin receptor PAR1, plays a central role both in the malignant invasion process of breast carcinoma metastasis and in the physiological process of placental implantation. The molecular mechanism underlying PAR1 involvement in tumor invasion and metastasis, however, is poorly defined. Here we show that PAR1 increases the invasive properties of tumor cells primarily by increased adhesion to extracellular matrix components. This preferential adhesion is accompanied by the cytoskeletal reorganization of F-actin toward migration-favoring morphology as detected by phalloidin staining. Activation of PAR1 increased the phosphorylation of focal adhesion kinase and paxillin, and the induced formation of focal contact complexes. PAR1 activation affected integrin cellsurface distribution without altering their level of expression. The specific recruitment of $\alpha_v\beta_5$ to focal contact sites, but not of $\alpha_v\beta_3$ or $\alpha_v\beta_6$, was observed by immunofluorescent microscopy. PAR1 overexpressing cells showed selective reciprocal co-precipitation with $\alpha_v\beta_5$ and paxillin but not with $\alpha_v\beta_3$ that remained evenly distributed under these conditions. This co-immunoprecipitation failed to occur in cells containing the truncated form of PAR1 that lacked the entire cytoplasmic portion of the receptor. Thus, the PAR1 cytoplasmic tail is essential for conveying the cross-talk and recruiting the $\alpha_v\beta_5$ integrin. While PAR1 overexpressing cells were invasive in vitro, as reflected by their migration through a Matrigel barrier, invasion was further enhanced by ligand activation of PAR1. Moreover, the application of anti-$\alpha_v\beta_5$ antibodies specifically attenuated this PAR1 induced invasion. We propose that the activation of PAR1 may lead to a novel cooperation with the $\alpha_v\beta_5$ integrin that supports tumor cell invasion.

* This work was supported in part by grants from the Ministry of Health, the Ministry of Science and the Arts, the Joint German and Israeli Research Program, the Middle East Cancer Consortium, the Israel Cancer Association, and the Israel Science Foundation of Science and Humanities (to R. B.-S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom all correspondence should be addressed. Permanent address: Dept. of Oncology, Sharrett Inst., Hadassah University Hospital, P.O. Box 12000, Jerusalem 91120, Israel. Tel.: 972-2-677-7563; Fax: 972-2-642-2794; E-mail: barshav@md2.huji.ac.il. Current address (during the academic year 2000–2001): Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-3971/3972; Fax: 617-432-3969; E-mail: rachel_barshavit@hms.harvard.edu.

1 The abbreviations used are: PAR, protease activated receptor; FAC, focal adhesion complex; FAK, focal adhesion kinase; ECM, extracellular matrix; TRAP, thrombin receptor-activating peptide; AS, antisense; FACS, fluorescence-activated cell sorting; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PTDc, fluorescein isothiocyanate–mAb, monoclonal antibodies; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TF, tissue factor; uPA, urokinase; uPAR, urokinase receptor.
tivation. Instead, they are activated by a specific cleavage within their extracellular N-terminal portion to unmask a new amino acid terminus, which serves then as an internal ligand for activation (14–18). Until now, four members of the PAR family have been identified and of these, three (PAR1, PAR3, and PAR4) have been established collectively as “thrombin receptors,” possibly serving as a redundant receptor system for the coagulation protease cellular response (14).

Since solid tumors are in close contact with ECM components, malignant cell invasion into the surrounding tissues is facilitated by mutual interactions that convey essential signaling cues to the cells (19, 20). These cell-ECM interactions are mediated by integrins, a family of adhesion receptors that mediate the attachment of the cell to both structural and matrix-immobilized proteins to promote cell survival, proliferation, and migration (21, 22). It is widely recognized that integrins perform a significant function in cellular invasion and metastasis (23–26). Non-ligated integrins are generally distributed diffusely over the cell surface with no apparent linkage to the actin cytoskeleton. However, ECM-bound integrins frequently cluster into specialized structures termed focal adhesion complexes (FACs), thus providing a convergence site for multiple signaling components (26, 27) and also physically linking the receptors to the actin filaments (28–30). The known signaling events that follow receptor clustering include tyrosine phosphorylation of proteins like focal adhesion kinase (FAK) and paxillin (31), as well as the recruitment of other FAC components like vinculin, talin, tensin, and p130 Cas (32–36). A growing number of studies indicate that signals driven by integrins act in concert with signals initiated by the G-protein-coupled receptors and with receptors for tyrosine kinase to promote the pathological tumor cell invasion process, on the one hand, and physiological activities like angiogenesis and wound healing (37, 38) on the other. The combined signals involved with the activation of focal adhesion proteins indicate that the cooperation between the signaling pathway takes place most likely within these FAC structures.

In the present work, we have studied the molecular mechanism of PAR1 involvement in tumor cell invasion. We show here that PAR1 modulates the invasive phenotype of melanoma cell lines, inducing the otherwise non-invasive cells to migrate effectively through Matrigel barriers. This process is accompanied by the increased adherence of the cells to various matrix components, actin stress fiber formation, and adhesion-triggered signaling, with no alteration of the cell surface integrin levels. We demonstrate now, for the first time, that PAR1 mediates these functions via selective cross-talk with the αβ₅ integrin to confer FAC formation, distinct signaling events, and cytoskeletal reorganization. Combined, these processes promote tumor cell invasion.

**EXPERIMENTAL PROCEDURES**

*Cells—SB-2 non-invasive human melanoma and A375-SM “supermetastatic” human melanoma cells (kindly provided by J. Fidler and M. Bar-Eli, Department of Cell Biology, University of Texas, M. D. Anderson Cancer Center, Houston, TX) were grown in 10% FCS-DMEM supplemented with 50 units/ml penicillin and streptomycin (Life Technologies, Inc.) and maintained in a humidified incubator with 8% CO₂ at 37 °C. The PAR1 stable transfectants, clone 13 and clone Mix L, were grown under the same conditions; for long term maintenance, these were supplemented also with 200 μg/ml G418 antibiotics. MCF-7 cells were maintained as previously described (3).*

*Cell Transfection—Cells were grown to 30–40% confluence and then transfected with 0.5–2 μg/ml plasmid DNA in FuGene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 10 days of selection, stable, transfected clones were established in medium containing 400 μg/ml G418. Antibiotic-resistant cell colonies were transferred to separate culture dishes and were grown in 200 μg/ml G418 medium. Forty-eight hours after transfection, transiently transfected cells were collected and tested by immunoprecipitation and analyses (see below).*

*Preparation of Truncated PAR1—Using polymerase chain reaction, we constructed a PAR-1 mutant protein truncated in its cytoplasmic tail after the amino acid Leu-369. As a template, we used PAR-1 cDNA (pCDNA3 vector. For cloning the reverse primer, TGGCTTAGAATCTATAGGGGTCCGTGTCACGACCT containing a STOP codon and an XbaI site. The amplified DNA fragment was subcloned using the polymerase chain reaction-blunt technique (Invitrogen) and confirmed by DNA sequencing. The insert was released from the vector by XbaI digestion and cloned into pCMV-FAS. To confirm the functional integrity of the constructs, wild type and mutant cDNAs were transiently expressed in 293 cells that were subsequently stained with a PAR-1-specific antibody (WEDE15, Immunotech, Cedex, France).

*Western Blot Analysis—Cells were solubilized in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (5 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) at 4 °C for 30 min. The cell lysates were subjected to centrifugation at 10,000 × g at 4 °C for 20 min. The supernatants were saved and their protein contents were measured; 50 μg of the lysates were loaded onto 10% SDS-polyacrylamide gels. After the proteins were separated, they were transferred to an Immobilon-P membrane (Millipore). Membranes were blocked and probed with an αβ₅ antibody (μg/ml amount of the appropriate antibodies as follows: anti-PAR1 thrombin receptor mAb, clone II aR-A (Biodesign Int.); anti-paxillin monoclonal antibody (mAb), clone 349 (Transduction Laboratories, Lexington KY); anti-human focal adhesion kinase, rabbit polyclonal IgG (Upstate Biotechnology Inc., Lake Placid, NY); anti-phosphotyrosine mAb, clone 4G10 (Upstate Biotechnology Inc.); anti-vinculin mAb (Transduction Laboratories). The antibodies were suspended in 1% BSA in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% Tween 20. After washes with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% Tween 20, the blots were incubated with antibodies conjugated to horseradish-peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) reagent using Luminol and p-cumaric acid (Sigma).*

*Immunoprecipitation—Cells were treated for 30–60 min with thrombin at a concentration of 1 NIH unit/ml of serum-free DMEM medium (0.5% BSA), and then lysed as described above. We used 400 μg of total protein for immunoprecipitation of αβ₅, αβ₅, paxillin, FAK, or both paxillin and FAK. All the antibodies were used at a concentration of 10 μg/ml. After overnight incubation, Protein A-Sepharose beads (Amerham Pharmacia Biotech) were added to the suspension (50 μl) that was subsequently rotated at 4 °C for 1 h. Elution of the reactive proteins was made by re-suspending the beads in protein 2X sample buffer (63 mM Tris-HCl, pH 6.8, 20% glycerol, 20% SDS, 0.01% bromphenol blue, 5% β-mercaptoethanol, 0.02 M dithiothreitol) and boiling for 5 min. The supernatant was loaded on a 10% SDS-polyacrylamide gel followed by the same procedure as in Western blotting.

*Matrigel Invasion Assay—We used blind-well chemotaxis chambers with 3-μm diameter filters. Polyvinylpyrrolidone-free polycarbonate filters with 8-μm pores (Costar Scientific Co., Cambridge, MA) were coated with basement membrane Matrigel (25 μg/filter) as described previously (39). Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, dried under a hood, and reconstituted with serum-free medium. In the upper compartment of the Boyden chamber, we placed 2–3 × 10⁶ cells suspended in DMEM containing 0.1% bovine serum albumin. As a chemoattractant, into the lower compartment of the Boyden chamber, we put 3T3 fibroblast conditioned medium. Assays were carried out in 5% CO₂ at 37 °C. After 2 h of incubation, we observed that more than 90% of the cells were attached to the filter. At this time, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed in DiQuick system (American Scientific Products) and stained with hematoxylin and eosin. Cells from various areas of the lower surface were counted. Each assay was performed in triplicate. For chemotaxis studies (a control of Matrigel invasion), the filters were coated with collagen type IV alone (5 mg/filter) to promote cell adhesion. Cells were added to the upper chamber and conditioned medium to the lower compartment.

*Adhesion Assay—The medium of cells grown in 10% FCS was replaced by DMEM with 0.5% BSA, and the cells were detached from the plate by treating with 0.05% trypsin in a solution of 0.02% EDTA in 0.01 M sodium phosphate, pH 7.4 (Biological Industries, Beit Ha’emek, Israel). After washing, 5 × 10⁵ cells/ml cells were resuspended in a serum-free DMEM medium (as above) and laid on 13-mm culture dishes pre-coated with either 100 μg/ml fibronectin or Th-1, a thrombin-de-
rived RGD (arginine-glycine-aspartic acid) peptide. After a 45-min incubation period to allow cell adhesion, the excess cells were washed away. The adhered cells were fixed to the plates with 4% formaldehyde in PBS, pH 7.4, for at least 2 h. After fixation, the plates were washed in 1% boric acid solution and the cells were stained with 1% methylene blue reagent (Sigma) in 1% boric acid for 30 min. After extensive washing with tap water, the methylene stain was eluted by the addition of 500 μl of 1 M HCl. The intensity of the color staining was measured by color spectrometry at a wavelength of 620 nm.

Immunofluorescence—Cells were plated on glass coverslips in 16-mm culture dishes; after the cells had grown to subconfluence, they were washed with PBS, permeobilized in 0.5% Triton-X100-containing 3.5% paraformaldehyde/PBS solution on ice for 2 min, and finally fixed with 3.5% paraformaldehyde/PBS for 20 min. Reactions with the appropriate antibodies were performed in room temperature for 60 min, after which the cells were washed extensively in PBS. The antibodies included the following: anti-αv,β3 mAb clone LM609, anti-αv,β5 clone P1F6, and anti-αv,β5 clone J6BS, (all from Chemicon Int.). After the 60-min incubation with the primary antibodies, followed by extensive washes in PBS, an additional 60-min incubation was carried out in the dark with secondary antibodies, goat-anti-rabbit or goat-anti-mouse IgG each conjugated with Cy-3 (Jackson Immunoresearch Laboratories) diluted 1:700. Labeling of filamentous actin by 1 μg/ml FITC-conjugated phalloidin (Sigma) was performed similarly. The labeled cells were visualized and photographed by fluorescence confocal microscopy (MRC-1024 confocal imaging system, Bio-Rad).

Flow Cytometry Analysis—The medium of cells grown in 10% FCS-DMEM was replaced by serum-free DMEM containing 0.5% BSA. Thrombin at a concentration of 1 IU/ml was added to the plates that were activated by incubation for 60 min. The plates were washed with PBS, and the cells were detached from the plates by treatment with 0.05% trypsin in a solution of 0.02% EDTA in 0.1M sodium phosphate at pH 7.4 (Biological Industries). After being washed twice in PBS, the cells were re-suspended in 200 μl of PBS and the appropriate antibodies were added to a concentration of 10 μg/ml. These reactions, performed at room temperature for 60 min, were followed by extensive washing in PBS. A 1-h incubation with a secondary antibody goat-anti-mouse IgG (Jackson Immunoresearch Laboratories) conjugated with FITC and diluted 1:500 was carried out in the dark. The treated cells were washed extensively, re-suspended in 100 μl of PBS, and analyzed by FACS.

RESULTS

Altering the Expression of PAR1 Affected Tumor Cell Invasiveness—In previous work (3), we showed that there is a direct correlation between PAR1 expression and the metastatic potential of primary tumor biopsies and tumor cell lines, as reflected by their in vitro potential to invade through a Matrigel barrier. In a physiological invading model system of placenta trophoblast implantation, we have also shown that PAR1 is part of the invasive program of trophoblast, as evaluated by their villi extension and matrix metalloproteinase synthesis. Here, to clarify how high levels of PAR1 may confer invasiveness, we transfected a non-invasive melanoma cell line (SB-2 cells) with PAR1 cDNA and compared the properties of the transfected cells to those of the highly invasive melanoma cell line A375SM. We used PAR1 cDNA under the control of the cytomegalovirus viral promoter in the pCDNA3 expression vector. We selected several stable clones that expressed high levels of PAR1, as evaluated by Western blot analysis (Fig. 1a) and Northern blot analysis (data not shown). The selected clones were then tested for their ability to invade through Matrigel-coated filters. Indeed, clones expressing high levels of PAR1 had an increased ability to invade the Matrigel layer, as compared with control clones transfected with empty vectors or SB-2 cells that had not been transfected at all (Fig. 1b). In addition, we observed that, whereas highly invasive A375SM cells invaded Matrigel coated membranes more efficiently than did non-metastatic cells (Fig. 1b, SB-2), activating the A375SM cells with PAR1 increased their ability to invade Matrigel at an even higher level (Fig. 1b, activ. A375SM). In addition, the invasiveness of PAR1-transfected cells was further increased when they were either activated by thrombin, as shown in two separate PAR1-transfected clones (Fig. 1b, clones 13 and Mix L), or when they were treated with the thrombin receptor-activating peptide (TRAP) that corresponds to PAR1 internal ligand SFLLRNR (data not shown).

Circulating tumor cells can invade into a new metastatic site only if they can adhere to the basement membrane. We analyzed the adhesion properties of cells suspended in a serum-free medium and then incubated for 60 min on plates coated with either fibronectin, a major component of the ECM, or with TH-1, an 11-amino acid peptide, corresponding to the thrombin RGD motif (40). Highly invasive A375 SM melanoma cells adhered strongly to both TH-1 and fibronectin; however, under the same conditions, the non-invasive SB-2 cells failed to adhere. We observed a marked increase in the adherence to both of these matrices of PAR1-transfected SB-2 cells (Fig. 2, a and b). The level of adherence of these PAR1 transfectants was directly correlated both with their level of PAR1 expression and with their ability to invade the Matrigel barrier. To assure that this increase in their adherence was actually caused by the presence of PAR1, we asked if reducing the expression of PAR1 in malignant cells would reduce the adhesion properties of these cells. To do this, we evaluated the effect of transfection by PAR1 antisense DNA on the adhesion properties of the invasive A375SM cells. We used a 462-base pair oligonucleotide fragment corresponding to the 5’ region of PAR1 that included part of the near promoter sequence and the coding region for the internal ligand. We cloned this DNA segment into pCDNA3 mammalian expression vector in an antisense orientation, selecting for stable clones expressing the plasmid bearing the PAR1 antisense DNA as compared with cells transfected by empty vectors or non-transfected control cells. Northern blot analysis (Fig. 2d) indicated that, whereas empty vector transfection (Fig. 2d, A), clones AS-3 (Fig. 2d, C) and AS-4 (Fig. 2d, D), which were transfected by the PAR1 antisense DNA, did exhibit reduced PAR1 expression. When we analyzed clones AS-3 and AS-4 for their adhesion properties, we found that the cell adherence properties to fibronectin (Fig. 2e) and to TH-1 (data not shown) of both of these clones were significantly lower than those of the A375 SM parental cells.

The organization of the cytoskeleton is critically influenced by adhesion interactions. To explore the effect of PAR1 activation on cytoskeletal reorganization, we plated PAR1-transfected cells (clone 13) and control non-transfected cells (SB-2 cells) on glass coverslips and then treated them with TRAP for various periods of time (Fig. 2e). After activation by TRAP, the cells were permeabilized, fixed, and stained with FITC-labeled phalloidin to detect filamentous actin (F-actin). Cytoskeletal reorganization was observed as early as 15 min after activation by TRAP (Fig. 2e). Thirty to 60 min after PAR1 activation, we observed a transition in the PAR1 transfecants from elongated spindle-like shapes to spreading, jellyfish-like structures. Ninety minutes to 2 h after activation, the cells became rounder and we observed the appearance of a ringlike bundle of actin filaments at the base of the cells (typical of migrating cells). These changes occurred more rapidly and were more dramatic in PAR1-overexpressing cells than they did in the non-transfected control cells. Altogether, these data show that the adhesive properties of tumor cells were affected by changes in PAR1 expression.
Integrin activation typically leads to the assembly of focal adhesion contacts; this takes place by phosphorylation on tyrosine leading to the recruitment of various signaling and structural molecules. FAK and paxillin are the most common signaling components of FAC that are phosphorylated upon integrin activation. To analyze the phosphorylation levels of FAK and paxillin in PAR1-transfected cells, we immunoprecipitated these proteins from cell lysates of either thrombin-activated or non-activated control cells. The immunoprecipitated proteins were blotted onto a nylon membrane and probed with anti-phosphotyrosine mAb to detect their phosphorylation levels. FAK and paxillin proteins from parental, non-invasive SB-2 cells exhibited low levels of phosphorylation (Fig. 3a). On the other hand, FAK and paxillin from PAR1 transfectant cells exhibited increased phosphorylation to high levels similar to those observed in the metastatic line A375 SM (Fig. 3a). By immunofluorescent analysis using anti-phosphotyrosine mAb followed by a Cy-3 fluorescence-labeled secondary antibody, we detected FAC formation as soon as 15 min following PAR1 activation, reaching a maximum phosphorylation levels...
Fig. 2. Altering PAR1 expression affected cell adhesion and actin fiber re-organization. Stable PAR1-transfected clones and PAR1 antisense selected clones were analyzed for their adhesive properties to substrates coated with fibronectin (a and c) or Th-1 RGD peptide (b). Cell adhesion was measured by Methylene blue staining of formaldehyde-fixed cells. The eluted stain was detected by spectrophotometry using a λ = 620 nm filter. The cells tested (a and b) were the same as described in Fig. 1. In addition, we show that in highly metastatic human melanoma A375SM cells stably transfected by PAR1 antisense cDNA (AS clone 4 and AS clone 3), reduced adhesion was observed (c) as compared with A375SM cells that were not transfected or that were transfected by an empty vector. These clones exhibited low PAR1 levels as shown by Northern blot analysis (d) of RNA samples from A375SM (A), A375SM cells transfected with vector only (B), AS clone 3 (C), and AS clone 4 (D). The data presented here are the averages of data from at least three replicate experiments. L32 is a ribosomal RNA that we have used as a housekeeping control gene for these experiments. e, SB-2 cells and PAR1 transfectant clone13 were subjected to actin staining by FITC-phalloidin after PAR1 activation by TRAP. Note that the PAR1 transfectants exhibited a more rapid change in actin fiber re-organization and cellular morphology than did the naive SB-2 cells.
after 60 min (Fig. 3b). Together, these data demonstrate that, following activation, overexpressed PAR1 is capable of initiating high levels of integrin signaling.

We characterized FAC assembly by immunofluorescent staining, using mAb anti-vinculin, anti-paxillin, and FAK polyclonal antibodies. Following activation, we observed some FAC formation in all of the cell types that we examined; however, the complexes in the activated PAR1 transfectants were far more distinct and larger than those that we observed in non-activated cells (Fig. 3c), in cells that had been transfected by empty vectors, or in cells that had not been transfected (data not shown). In the activated PAR1 clones, vinculin staining of FACs was intense; however, we also observed clear, although less intense, vinculin staining of FACs in activated non-transfected and mock-transfected cells. This may be explained by the fact that, rather than having a signaling function, vinculin functions mainly as a structural protein, and it has been reported to play a role in the maintenance of the FAC and adherence junctions (41). It has also been reported that both vinculin and talin are phosphorylated even under basal conditions (42).

The \(\alpha_\beta_5\) Integrin Is Specifically Recruited to FACs in Response to PAR1 Activation without Alteration of the Cell-surface Integrin Level—Having established that signaling was induced by PAR1 ligand activation, that also led to establishment of focal contacts, we asked whether altering the adhesive phenotype would be accompanied by de novo integrin expression. Here we used flow cytometry analysis carried out with a battery of anti-integrin antibodies directed against the \(\alpha_\beta_3\), \(\alpha_\beta_1\), and \(\alpha_\beta_5\) integrins. Following activation, we observed no significant differences between the cell-surface integrin profiles of the PAR1 transfectants and of the parental cells (Fig. 4a). Nevertheless, the fact that PAR1 activation did not alter integrin expression does not exclude the possibility of affinity modulation of the integrins in an inside-out manner.

We then asked which of these integrins would respond to PAR1 by participating in the induction of the cytoskeleton signaling events. We examined the cell surface integrins by immunofluorescent visualization before and after PAR1 activation. Although \(\alpha_\beta_3\) and \(\alpha_\beta_1\) (Fig. 4b, B and C) are distributed diffusely over the cell surface both before and after PAR1 activation, after PAR1 activation we found that \(\alpha_\beta_5\) was localized to distinct sites of FACs (Fig. 4b, D). However, we only detected \(\alpha_\beta_5\) within the focal contacts in the activated PAR1-overexpressing cells (Fig. 4b, E) but not in the PAR1-transfected cells prior to PAR1 activation cells (Fig. 4b, A), nor in the mock transfectants or in the parental non-transfected cells (data not shown). Based on our results, we hypothesized that...
FIG. 4. PAR1 activation did not alter the levels of integrin expression but did induce α5β3 recruitment to FACs. a, integrin expression levels were measured by flow cytometry in SB-2 naive cells and in PAR1 clone 13, each activated with thrombin at a concentration of 1 unit/ml. The levels of α5β1 (A and B), αvβ3 (C and D), αvβ5 (E and F), and the integrin αv chain (G and H) were detected by incubating the cells with the appropriate specific mAb, followed by incubation with FITC-labeled anti-mouse IgG. The white peaks correspond to the expression levels of control secondary isotype-specific mouse IgG antibodies. Note that no significant changes were observed in the levels of any of the integrins examined. b, the distribution of integrins was detected by immunofluorescent staining (upper panel). Cy-3 red fluorescence was visualized by confocal microscopy. The lower panel shows the same cells as in the upper panel, but visualized by phase-contrast microscopy. Non-activated (NA) PAR1 clone 13, stained by anti-α5β3 mAbs revealed a diffused pattern (A). After activation by TRAP, the integrin α5β3 was detected in a random perinuclear position (B); the integrin αvβ5 was randomly scattered over the cell membrane (C); the integrin αvβ5 was localized to distinct “spikes” of focal adhesion contacts (D).
the αβ₃ integrin would respond to signals conveyed by the activated PAR1. It seemed that αβ₃ was specifically recruited to the focal adhesion contacts, where it played a major role in the reorganization of the cytoskeleton. Our hypothesis was confirmed by the results of the following reciprocal co-precipitation experiments. We analyzed the co-precipitation of paxillin with either αβ₃ or with αβ₁ in cell lysates of naive SB-2 cells and of the stable PAR1 transfectant clone 13 that was either thrombin-activated or not. The blotted membranes were incubated with the mAb of the anti-β₃ subunit. As we expected, in the parental cells, we found only basal levels of paxillin precipitation with either of the two integrins (Fig. 5, a and b).

In the PAR1 clone 13, we found that paxillin co-precipitated with αβ₃ at a low level, and that level was not increased significantly by thrombin activation of PAR1 (Fig. 5b). However, in PAR1 clone 13, we did find a high level of co-precipitation of αβ₃ with paxillin, and that level was significantly increased by thrombin activation of PAR1. We also analyzed co-immunoprecipitation of paxillin and FAK from cell lysates of SB-2 cells and from the stable PAR1 transfectant clone 13, both of which were thrombin-activated or not. Again, the blotted membranes were incubated with the mAb of anti-β₃ subunit. As we expected, there was no co-immunoprecipitation in the parental cells, whether or not they were activated; however, there was a significant level of co-precipitation of β₃ subunit in the PAR1 clone 13, that was greatly increased upon activation by thrombin (Fig. 5c). When, instead of anti-αβ₃, we used anti-αβ₁ to probe the same blot, we found no evidence of the β₃ subunit (data not shown). These results indicate that αβ₃ and the typical signaling molecules, paxillin and FAK, were tightly associated and thus co-precipitated. We found that this kind of association was likely to occur within focal adhesions rather than in other cellular compartments, as demonstrated by the induced assembly and signaling of FACs. Furthermore, this association appeared to be labile and seemed to occur in response to PAR1 activation, indicating that the αβ₃ integrin was present within newly assembled FACs. Our data do not exclude the possibility that αβ₃ is present on the cell surface. Our data do suggest, however, that the αβ₃ integrin probably does not cooperate with PAR1-specific signaling to induce the cellular responses described here.

To substantiate the cooperative cross-talk and the recruitment of αβ₃ following PAR1 activation, we used a truncated form of PAR1, consisting of the extracellular and seven transmembrane domains but lacking the entire cytoplasmic portion of the receptor, and compared its function to the intact receptor. We carried out these experiments in MCF7 cells, which are non-invasive cells that naturally express very low levels of PAR1. These parental cells were transiently transfected with cDNA coding for either the intact PAR1 or truncated PAR1; 48 h after transfection, the transient transfectants were either activated or not and then subjected to immunoprecipitation analysis as described above. In lysates of the MCF7 PAR1 transient transfectants, we detected high levels of co-precipitation of FAK, paxillin, and αβ₃ after PAR1 activation by thrombin but not without activation. In lysates of truncated PAR1 transfectants, we observed no co-precipitation of FAK with paxillin, and αβ₃, regardless of whether the cells were thrombin activated or not (Fig. 5d, upper panel). In the PAR1 transfectants, tyrosine phosphorylated paxillin co-precipitated with αβ₃ and the level of this precipitation increased following thrombin activation of PAR1; in truncated PAR1-transfected cells, we detected only minor levels of phosphorylated paxillin with or without thrombin activation (Fig. 5d, lower panel). As seen by the results of the flow cytometry (FACS) analysis (Fig. 5e), the failure to immunoprecipitate FAK by anti-αβ₃ in PAR1-truncated transfectants did not result from the inability to express properly on the cell surface. Transfectants of either PAR1 (Fig. 5e, A) or truncated PAR1 (Fig. 5e, B) showed cell surface expression of the truncated receptor protein as determined by flow cytometry (FACS) analysis (Fig. 5e). Using anti-PAR1 WEDE15 mAbs, we found similar levels of expression in both the PAR1 (Fig. 5e, A, second peak) and the truncated PAR1 (Fig. 5e, B, second peak) transfectants, relative to the expression levels in naive cells (Fig. 5e, A and B, first peaks). We obtained similar results when we compared the levels of expression of the transfectants (Fig. 5e, C and D, second peaks) to those of empty vector-transfected cells (Fig. 5e, C and D, first peaks). The results of these experiments strongly support the notion that following activation the PAR1 cytoplasmic tail recruits and activates the αβ₃ integrin. That the PAR1 molecule participates in other signaling activities is supported by our finding that, although she was phosphorylated in the presence of the full-length activated PAR1, this was not the case in the presence of the activated truncated PAR1 (data not shown). We conclude that, although, like the full-length PAR1, the truncated PAR1 is expressed and assembled on the cell surface, unlike the full-length PAR1, the truncated PAR1 is incapable of carrying out PAR1 signaling. To ascertain that in fact the αβ₃ integrin may cooperate with PAR1 during tumor invasion, we asked whether neutralizing the activity of the αβ₃ integrin would affect the invasive properties of the highly invasive A375 SM cells. A375 SM cells were activated or not with 1 unit/ml thrombin; the activated cells were then either not treated at all or treated with anti-αβ₃-blocking mAbs antibodies (clone P1F6) or with nonspecific IgG. The cells were then subjected to a Matrigel invasion assay. As one can see, PAR1 activation further induced the invasive properties of the cells by 60% while the addition of anti-αβ₃ antibodies attenuated this induction; the addition of a non-related IgG led to no significant effect (Fig. 5f).

**DISCUSSION**

In this study, we have shown that changes in the expression of PAR1 in a cell affect its invasive capabilities. These changes come about through the specific recruitment of the αβ₃ integrin, through cytoskeletal reorganization, and through distinct signaling at FACs. The fact that PAR1 alters the invasive properties of tumor cells reinforces our initial observations that PAR1 expression correlates with the invasive potential of both the malignant invasion processes of breast carcinoma (3) and the physiological invasion processes of placenta trophoblast implantation (3) emphasizing the central role of PAR1 during invasion. The on-going process of invasion by cells is characterized by extensive proteolytic remodeling, in part by serine proteases, of the tumor microenvironment (1, 2). Serine proteases also serve as ligands for several cell-surface receptors, among which is uPAR, which, through binding uPA, efficiently converts plasminogen to plasmin (11). TF is another protease cell surface receptor that binds factor VII, thereby initiating the coagulation pathway during perivascular hemostasis (43). It is interesting that, in addition to their involvement in hemostasis, these receptors are also implicated as central players in tumor progression and metastasis (6–10). The extracellular proteolytic activation of factor VII by TF is also responsible for the generation of thrombin from circulating plasma prothrombin (44, 45). In fact, thrombin production is probably the direct result of disseminated overactivation of the coagulation system, a widely described pathology among cancer patients (46). The abundant localization of either soluble or immobilized thrombin in the vicinity of the tumor milieu enables the excessive activation of PAR1 and the subsequent cellular response during invasion. In fact, although the repertoire of signaling
FIG. 5. Activation of full-length PAR1 but not of truncated PAR1 led to the co-precipitation of αvβ5 with paxillin and FAK and reduced invasiveness in the presence of anti-αvβ5 antibodies. Co-precipitation of paxillin with αvβ5 (a) or with αvβ3 (b) was measured in cells lysates of naive SB-2 cells (C) and of a stable PAR1 transfectant clone 13 that was either thrombin-activated (A) or not (B). c, paxillin and FAK were immunoprecipitated from cell lysates of SB-2 cells that had been thrombin-activated (A) or not (B) and from the stable PAR1 transfectant clone 13 that was either thrombin-activated (C) or not (D). The blotted membrane was incubated with anti-β5 subunit mAb. d, non-invasive MCF7 cells, naturally expressing very low levels of PAR1, were transfected with cDNA expression vectors coding either for PAR1 or for truncated PAR1. In lysates of the PAR1 transfectants, co-precipitation of FAK and paxillin was detected after PAR1 activation by thrombin (B) but not without activation (A). In lysates of truncated PAR1 transfectants, no co-precipitation of FAK with paxillin with αvβ5 was observed regardless of whether the cells were thrombin-activated (D) or not (C). Tyrosine-phosphorylated paxillin co-precipitated with αvβ5 (lower panel) in PAR1-transfected cells (A); the level of this precipitation increased following thrombin activation of PAR1 (B); in truncated PAR1-transfected cells, only minor levels of phosphorylated paxillin were detected with (D) or without (C) thrombin activation. e, PAR1 expression levels were measured by FACS analysis using anti PAR1 mAb (WEDE15, Immunotech, Cedex, France), followed by incubation with FITC-labeled anti-mouse IgG. The analysis was carried out on MCF7 cells following transfection by DNA coding for the full-length PAR1 (A and C) or for the truncated PAR1 (B and D). Their levels were compared with non-transfected cells (first peak, B and A). This is true also when PAR1 or truncated PAR1 expression was measured relative to empty vector-transfected cells (first peak, C and D, respectively). f, A375SM cells were activated with 1 unit/ml thrombin (B–D) or not (A). The activated cells were then treated with 20 μg/ml either anti-αvβ5-blocking mAbs (C) or nonspecific IgG (D). The treated cells were then subjected to a Matrigel invasion assay. The data presented here are the averages of data from at least three replicate experiments. One hundred percent invasion by the metastatic cells corresponded to 48 ± 6 invading cells as compared with 17 ± 1.5 invading cells by SB-2 non-metastatic cells (data not shown).
PAR1 Activation Promotes Integrin Signaling and Invasion

pathways is limited, it can be harnessed to integrate the information obtained from multiple receptors for a wide range of cellular responses. Here we have presented evidence showing that the overexpression of PAR1 increases the invasiveness of melanoma cells (Figs. 1b and 5f) and is also associated with an increase in the adhesion properties of the cell (Fig. 2, a–c). The activation of PAR1 resulted in the phosphorylation of the focal adhesion proteins FAK and paxillin that are typical of integrin signaling (Fig. 3a). Although the levels of the cell surface integrins were not affected (Fig. 4a), there was notable change in their mode of distribution. In particular, in response to PAR1 activation, the integrins αvβ3 and αvβ1 were found to be distributed diffusely but, in contrast, we found that the integrin αvβ5 was uniquely recruited to the sites of focal adhesion contacts (Fig. 4b).

It is well established that there is cooperation between integrins and other cell surface receptors, and further, that this cooperation may operate at several levels (47–50). Physical interactions between the extracellular domains of integrins and non-integrin receptors may result in mutual or sequential activation. For example, the results of several parallel studies demonstrate a physical link of uPAR with the integrin α5β1 in keratinocytes (51), with the β3 integrin Mac-1 in leukocytes (52), and with αvβ6 in breast cancer (53). The interaction of uPAR with integrin β3 has also been shown to involve the functional cooperation of integrins with cell surface receptors via caveolin in a manner dependent on the conformational state of the receptors (54, 55). Alternatively, the activation state of integrins can be modified in an “inside-out” manner. Internal signals conveyed by intersecting cascades react with the cytoplasmic domain of the integrin β subunit and thereby increase the affinity to their ligands of the extracellular portion of the integrin. Activation of G-protein-coupled receptor initiates a signal transmission through the C-terminal cytoplasmic domain of the receptor that leads to the assembly of adaptor proteins, non-receptor tyrosine kinases, and small G-proteins. Signals that are transduced in forking pathways, like Ras-Raf-mitogen-activated protein kinase and phosphatidylinositol 3-kinase-Akt/PKB, are also largely shared by integrin and thymidine kinase receptors. In endothelial cells (56), astrocyte cell rounding (57), and neutrophil retraction (58), cytoskeletal responses to thrombin are known to involve the activation of the Ras-dependent ERK1/2 mitogen-activated protein kinase pathway during gap formation. These responses have been found to be Rho-dependent and require Rho-specific guanine nucleotide exchange factors (57, 59). More specifically, the Rho-dependent pathway controls barrier maintenance and stress fiber formation while Rac induction and myosin light chain kinase activation are both implicated in barrier dysfunction (56). Together, these facts imply that integrin-related signaling can be intersected by PAR1 signaling at the intracellular level.

Thrombin contains a cryptic RGD epitope that can potentially be recognized by integrins (40). The transient binding of thrombin to its receptor, prior to receptor cleavage, may serve as an RGD-exposing event that enables integrin binding during PAR1 activation. Thus, it seems that, at least theoretically, cooperation between PAR1 and the vitronectin receptor αvβ3 may occur at the extracellular level. Using a truncated PAR1 construct that lacks the entire cytoplasmic tail domain, we demonstrated here that it was the cytoplasmic portion of the PAR1 molecule that was responsible for cooperation with the αvβ3 integrin. We found that the truncated PAR1 was unable to transmit intracellular signals, and therefore, was unable to recruit αvβ3 and to initiate the typical integrin-associated signals. The other vitronectin receptor, αvβ5, has been widely implicated in both angiogenesis and melanoma cell invasion and metastasis (60, 61). Nevertheless, many tumor cells that lack αvβ5 can still readily metastasize (62). In cells that express both αvβ3 and αvβ5, αvβ3 is constitutively capable of inducing cell spreading and migration, while αvβ3 cannot promote cell spreading and migration without an additional exogenous soluble factor (60, 63). Based on our results, we propose that during the invasion process αvβ3 is the dominant integrin involved in PAR1-ECM signaling interactions. This is consistent with previous suggestions (64) that αvβ3 has a role in mediating human keratinocyte locomotion. Filardo et al. (36) also showed that αvβ3, as the sole integrin expressed in melanoma cells, could promote cell spreading and migration in cooperation with insulin-like growth factor signaling. It has also been postulated that αvβ3-mediated cell migration is protein kinase C θ-dependent (66). Whether, as has been shown for endothelial cell migration (67), PAR1-mediated association of αvβ3 during tumor invasion is under the regulation of protein kinase C θ associated to TAP20 (theta-associated protein) remains to be determined.

The adhesion of tumor cells to the basement membrane is an essential step in the process of invasion. In contrast to the passive, non-active nature of non-malignant cells, the dynamic nature of tumor cell adherence to the underlying ECM precedes matrix degradation and migration. The interactions of the cell matrix involve the activation of integrins as well as the initiation, through focal adhesion structures, of signaling cascades that lead to cytoskeletal reorganization. This has been shown to be the case during tumor progression where TF supports cell adhesion, migration, and spreading through the action of the cytoplasmic portion of the TF molecule (10). The interaction of uPA with its cell surface receptor uPAR is necessary for vitronectin-dependent human pancreatic carcinoma (FG) cell adhesion and migration mediated via the αvβ3 integrin (65). The convergence point of the PAR1 and the αvβ3 signaling pathways is not yet known and is currently under study in our laboratory. Nevertheless, the data that we have presented here suggest that this unique mode of cooperation specifically promotes the invasive properties of tumor cells. We believe that the PAR1 and the αvβ3 signaling pathways that we have studied here may prove to be crucial for other PAR1 functions in vascular biology and embryonic development.

Acknowledgements—We thank Prof. Israel Vlodavsky for helpful discussions, Dr. Xiao-Ping (Merek Research Laboratory, West Point, PA) for providing us with the full-length PAR1, and F. R. Warshaw-Dadon for editorial revisions of the text.

REFERENCES

1. Migliatti, P., and Rifkin, D. B. (1993) Physiol. Rev. 73, 161–195
2. Johnsen, M., Lund, L. R., Romer, J., Almholt, K., and Dano, K. (1998) Curr. Opin. Cell Biol. 10, 667–671
3. Even-Ram, S., Uziel, B., Cohen, P., Girisu-Granosovsky, S., Maoz, M., Ginsburg, Y., Reich, R., Vlodavsky, I., and Bar-Shavit, R. (1998) Nat. Med. 4, 909–914
4. Cecconi, V. A., and Binder, B. R. (1999) Semin. Thromb. Hemost. 25, 183–197
5. Kurschat, P., Zigrino, P., Nischt, R., Breitkopf, K., Steurer, P., Klein, C. E., Krieg, T., and Mauch, C. (1999) J. Biol. Chem. 274, 21056–21062
6. Al-Mondhiry, H. (1975) J. Clin. Invest. 54, 1841–1893
7. Shoji, M., Hancock, W. W., Abe, K., Mico, C., Casper, K., Baine, R. M., Wilcox, J. N., Danave, I., Dillehay, D. L., Matthews, E., Contrino, J., Morrissey, J. H., Gordon, S., Edington, T. S., Kudryk, B., Kreutzer, D. L., and Rickles, F. R. (1981) Am. J. Pathol. 102, 399–411
8. Bromberg, M. E., Konigsberg, W. H., Madison, J. F., Pawashe, J. F., and Garen, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8205–8209
9. Mueller, B. M., and Ruff, W. (1998) J. Clin. Invest. 101, 1372–1378
10. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruff, W. (1998) J. Cell Biol. 40, 1241–1253
11. Vassalli, J. D., Sappino, A. P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1072
12. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) Science 273, 1551–1555
13. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheres, D. A. (1994) Cell 79, 1157–1164
14. Kahn, M. L., Hannes, S. R., Botka, C., and Coughlin, S. R. (1998) J. Biol. Chem. 273, 23290–23296
Tumor Cell Invasion Is Promoted by Activation of Protease Activated Receptor-1 in Cooperation with the \( \alpha_v\beta_5 \) Integrin
Sharona Cohen Even-Ram, Miriam Maoz, Elisheva Pokroy, Reuven Reich, Ben-Zion Katz, Paul Gutwein, Peter Altevogt and Rachel Bar-Shavit

*J. Biol. Chem.* 2001, 276:10952-10962.
doi: 10.1074/jbc.M007027200 originally published online January 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M007027200

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/276/14/10952.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 67 references, 31 of which can be accessed free at [http://www.jbc.org/content/276/14/10952.full.html#ref-list-1](http://www.jbc.org/content/276/14/10952.full.html#ref-list-1)