Evidence of Key Role of Cdk2 Overexpression in Pemphigus Vulgaris*[S]

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The pathogenesis of pemphigus vulgaris (PV) is still poorly understood. Autoantibodies present in PV patients can promote detrimental effects by triggering altered transduction of signals, which results in a final acantholysis. To investigate mechanisms involved in PV, cultured keratinocytes were treated with PV serum. PV sera were able to promote the cell cycle progression, inducing the accumulation of cyclin-dependent kinase 2 (Cdk2). Microarray analysis on keratinocytes detected that PV serum induced important changes in genes coding for one and the same proteins with known biological functions involved in PV disease (560 differentially expressed genes were identified). Then, we used two different approaches to investigate the role of Cdk2. First, small interfering RNA depletion of Cdk2 prevented cell-cell detachment induced by PV sera. Second, pharmacological inhibition of Cdk2 activity through roscovitine prevented blister formation and acantholysis in the mouse model of the disease. *In vivo* PV serum was found to alter multiple different pathways by microarray analysis (1463 differentially expressed genes were identified). Major changes in gene expression induced by roscovitine were studied through comparison of effects of PV serum alone and in association with roscovitine. The most significantly enriched pathways were cell communication, gap junction, focal adhesion, adherens junction, and tight junction. Our data indicate that major Cdk2-dependent multiple gene regulatory events are present in PV. This alteration may influence the evolution of PV and its therapy.

Pemphigus is traditionally considered as a group of autoimmune blistering diseases affecting skin and mucous membranes. It is characterized by the presence of autoantibodies against the intercellular substance of keratinocytes (1). Although several subtypes of pemphigus can be recognized on the basis of both clinical and laboratory criteria, two main forms have been described so far, namely pemphigus vulgaris (PV)[3] and foliaceus (PF). The best characterized autoantigens of PV and PF are the desmosomal proteins Dsg3 and Dsg1 (desmoglein 3 and 1), respectively (2), belonging to the cadherin family of calcium-dependent cell adhesion molecules (3). The common denominator of all types of pemphigus is the disruption of the intercellular bridges among keratinocytes, termed acantholysis. Morphological changes occurring in PV acantholytic cells have been reproduced both in cultured keratinocytes and *in vivo* in neonatal mice (4, 5). However, it is not yet clear whether PV IgG can experimentally mimic the disease on its own, since the action of serum factors other than IgG in PV blistering has been recently emphasized (6, 7). Despite years of research on PV pathophysiology, to date precise molecular mechanisms underlying acantholysis have not yet been fully understood. Until recently, it was believed that disruption of cell-cell contacts resulted from the simple interaction of PV autoantibodies with Dsgs by steric hindrance (8, 9). However, today this model appears untenable, since (*a*) autoimmunity in PV is not just restricted to adhesion molecules (10), (*b*) serum factors other than IgG could play a role in PV pathogenesis (11), and (*c*) a number of signal transduction cascades seem to be crucially involved in acantholysis (12, 13). With regard to this, an emerging concept is that phosphorylation events could be impaired in PV. Consistently, activation of signaling through both protein kinase C isoforms and p38 mitogen-activated protein kinase have been proposed to initiate acantholytic changes by inducing desmosome disassembling and cytoskeleton reorganization (12, 14).

Enzymatic phosphorylation has proven to be the most rapid reversible post-translational modification of proteins affecting their activity, localization, and stability. Phosphorylation events are controlled by protein kinases, which can regulate the onset and transition through mitosis (15). Thus, PV sera may influ-

ence the cell cycle progression of keratinocytes. One of the central kinases involved in G_1 regulation is the Cdk2 (cyclin-dependent kinase 2), an essential component of the cell cycle machine, which can be altered in several diseases, such as cancer and gestational trophoblatic disease (16–19). To date, no precise correlations between PV and keratinocyte cell cycle progression have been established. The goal of the present study was to investigate the pathogenic molecular events involved in PV.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Polyclonal IgGs against Perk, phospho-Perk, Akt, phospho-Akt, Ask1, αPAK, phospho-αPAK, Jak1, Cdk2, PKR, phospho-PKR, phospho-p38, CK1, Dsg1, STE-20, β-actin, pancytokeratin, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nitrocellulose membranes were purchased from Invitrogen; reagents for enhanced chemiluminescence and films were from Amersham Biosciences. All reagents used for protein extraction and cell cultures were obtained from Sigma, except keratinocyte growth medium and antibiotics/antimicotic, purchased from Invitrogen. Roscovitine was obtained from Calbiochem.

Patients and Sera—Sera of patients with untreated active PV ($n = 4$, named PV1–PV4) and from two volunteers without any skin disease ($n = 2$, NS1 and NS2) have been used for the experiments. Informed consent was obtained from all participants, who have collaborated in our studies for 30 years (20, 21). The diagnosis of PV was made based on the results of both clinical and histological examinations, according to well established criteria (22). PV patient skin biopsies used for diagnosis served as samples for immunohistochemical analyses. In pilot experiments ($n = 2$), PV IgG was purified from sera by ammonium sulfate precipitation followed by affinity chromatography on Protein G (HiTrap; Amersham Biosciences). IgG fractions were dialyzed against PBS and sterile filtered, and $CaCl_2$ was added to a final concentration of 0.5 mM. The presence of autoantibodies in sera was determined by indirect immunofluorescence using monkey esophagus as substrate (values above 1:40 of circulating anti-intercellular substance antibodies were considered as positive) and confirmed by Western blotting against Dsg1 and Dsg3.

Cell Cultures and Treatments—We have exposed cultured cells to PV patient sera as proposed originally by Swanson and Dahl (23). On the basis of both our preliminary experiments with PV IgG and other studies (22–24), we preferred to set experiments in the presence of PV serum instead of isolated IgG. Primary human keratinocytes were isolated from tongue specimens of patients undergoing glossoplasty. Each specimen was rinsed in phosphate-buffered saline (PBS) and submerged for 30 min in Dulbecco's modified Eagle's medium with 10% antibiotics/antimycotic. The epithelial sheet was partially separated from the connective tissues after overnight incubation in dispase at 4 °C. Keratinocytes were isolated by treatment in 0.25% trypsin for 30 min at 37 °C and plated in modified FAD (F-12 and Dulbecco's modified Eagle's medium) medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and fungizone (2.5 μg/ml) (25).

FAD was than changed with serum-free keratinocyte growth medium. HaCaT cells, a human immortalized keratinocyte cell line capable of forming normally differentiated epidermis (26), were maintained in Dulbecco's modified Eagle's medium enriched with 10% fetal bovine serum and 1% antibiotics/antimycotic, as described (22, 27). Primary keratinocytes and HaCaT were growth at 37 °C in an atmosphere humidified with 5% CO_2. At the time of the experiment, cells were seeded on 35-mm Petri plastic dishes and growth to confluence.

Cell Cycle Analysis—In order to study major effects of PV serum on the keratinocyte cell cycle, HaCat keratinocytes were starved, 16 h after plating, with 0.5% of fetal bovine serum for 48 h. The starvation method has been set by the use of a colorimetric immunoassay based on the measurement of bromodeoxyuridine incorporation during DNA synthesis (cell proliferation enzyme-linked immunosorbent assay; Roche Applied Science). Synchronized cells have been treated with PV serum (30%) and normal serum (30%) for 24 h. Fresh cells were pretreated with 100 g/ml RNase for 30 min at 37 °C and stained with 20 g/ml propidium iodide before flow cytometric analysis (FACSCalibur; BD Biosciences). Cell distribution in the G_1, S, and G_2/M phases of the cell cycle was calculated from the resulting DNA histogram with BD CellQuest software. Results are representative of six separate experiments in duplicate. Data are expressed as mean $\pm$ S.D. Differences were assessed by t test, and a p value of <0.05 was considered to be significant.

Western Blot Analysis—50-μg cytosolic extracts of sample were separated by 8–12% SDS-PAGE and transferred to nitrocellulose membranes as described (25–27). Primary antibodies and horseradish peroxidase-conjugated species-specific IgG were diluted 1:1,000 and 1:10,000, respectively. Bound antibodies were detected by an enhanced chemiluminescence immunodetection system. To ascertain that blots were loaded with equal amounts of protein lysates, filters were stripped and subsequently incubated with a polyclonal antibody against β-actin protein. Band intensity was quantified by scanning films with the LKB densitometry analysis system (27).

In Vitro Model of PV Using Cultured Keratinocytes—The standard protocols for treatment of keratinocyte monolayers with pemphigus serum were followed (23, 24, 28). For reproducing the *in vitro* model of PV on cell cultures, keratinocytes were incubated with 30% sera from PV patients and controls for 24, 48, or 72 h. In some cases, cells were exposed transiently to PV serum, and the medium was replaced after selected time points (see Fig. 1, *e* and *f*, and Fig. 2, *a* and *c*). Specific changes in cytoskeleton organization, such as retraction of keratin filaments from cell borders onto nucleus and detachment of keratinocytes from one another evaluated by staining of desmosomal components, such as Dsg3, both have been considered as the hallmarks of pemphigus acantholysis *in vitro* (23, 24, 28).

siRNA Treatment—Cdk2-directed siRNA pool (L-003236) and negative control pool (D-001206-13-05) were purchased from Dharmacon, Inc. (Lafayette, CO). The siRNAs were transfected at a final concentration of 100 nM using Dharmafect 1 according to the manufacturer's recommendations. The siRNAs were incubated with glass coverslip-cultured HaCaT cells overnight. Afterward, cells were treated with 30% PV and 30% control sera for both 24 and 48 h and then formalin-fixed

for immunofluorescence studies or lysed as described below for Western blot analyses. The efficiency of transfection was monitored by using siglo cyclophilin B as control (D-001610-01-05) as well as by Western blot for Cdk2 expression.

In Vivo Model of PV by Passive Transfer of Sera in Neonatal Mice—This study was conducted according to the Guidelines for Animal Experiments of the American Heart Association and rules of the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985) and approved by the institutional care of experimental committees. All efforts were made to minimize the number of animals used and their suffering. Quality standards of laboratories at the University of Naples (Italy) are in accordance with rules established by the Italian Ministry of Health and the European College of Laboratory Animal Medicine. As described by Amagai *et al.* (30) and Li *et al.* (31), 8–12-h-old neonatal BALB/c mice were injected intraperitoneally through a 30-gauge needle with 150 μl of 10-fold concentrated sera. Some pups were pretreated with 100 μg/g roscovitine (32) in 20 μl of PBS ($n = 6$) or 20 μl of PBS alone as a control ($n = 6$). After 12 h, the animals were subjected to gentle scraping of the lumbar skin to induce eventually the Nikolskiy phenomenon, and then, 24 h after injection, mice were evaluated for macroscopic blistering. Biopsies were taken from perilesional areas, if any, by using a standard 5-mm skin biopsy punch. After rinsing in PBS, these skin samples were either stored at $-80\,°C$ and subsequently subjected to RNA extraction or immediately fixed in 10% formalin for regular histological analysis.

Immunofluorescence, Immunohistochemistry, and Histology—Immunofluorescence studies were carried out as described previously (24, 28). Briefly, cells were grown on glass coverslips and incubated with normal or PV sera. After washing with PBS, cells were fixed and permeabilized in paraformaldehyde solution (3% paraformaldehyde in PBS containing 0.1% Triton X-100) for 20 min at room temperature and then washed three times in PBS containing 2% bovine serum albumin to block nonspecific sites. Samples were incubated with the appropriate primary antibody (1:10) for 1 h on ice, washed in bovine serum albumin/PBS, and fixed in 10% formalin for 10 min. Finally, cells were exposed to species-specific antibodies (1:100) conjugated to fluorescein isothiocyanate or Texas Red (Dako Denmark A/S). In some experiments, cells were directly incubated with anti-human IgG to investigate deposits of serum antibodies in the intercellular space. Specimens were examined with a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY) at $\times$400 magnification, and fluorescence images were acquired with an Evolution VF fast digital camera (MediaCybernetics, UK). For histologic analysis, neonatal mouse tissues were fixed in 10% formalin, processed for paraffin sectioning, and stained with hematoxylin and eosin by routine methods (33, 34). Formalin-fixed, paraffin-embedded skin biopsies from pemphigus patients were subjected to immunohistochemical analysis as described in detail (33, 34).

Morphometric Analysis of Cell-Cell Detachment—The extent of cell detachment (acantholysis) in cell monolayers was measured by modifying previously published protocols (35). Cells were subjected to immunofluorescence procedures, and the images of 10 representative microscopic fields were recorded.

The percentage of acantholysis in each field observed by immunofluorescence was computed (Adobe Photoshop) by subtracting the percentage of the area covered by the cells from the total areas of the microscopic field, taken as 100%.

RNA Extraction, Microarray Analysis, and Reverse Transcription-PCR—Tissues were snap-frozen in liquid nitrogen and stored at $-80\,°C$ until use. Total RNAs were extracted from mouse tissues and cultured cells using Trizol^{TM} solution (Invitrogen), according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (virtual presence of sharp 28 and 18 S bands) and spectrophotometry (36, 37). 5 μg of total RNA was used as starting material for the cDNA preparation. The first and second strand cDNA synthesis was performed using the GeneChip One-Cycle cDNA Synthesis Kit (Affymetrix) according to the manufacturer's instructions. Labeled cRNA was prepared using the GeneChip IVT Labeling Kit (Affymetrix) according to the manufacturer's instructions. Following the IVT reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen) (36, 37). 15 μg of cRNA was for hybridization onto the Affymetrix Mouse Genome 430A 2.0 and Affymetrix Human Genome U133 2.0 probe array cartridge. The washing and staining procedure was performed in the Affymetrix Fluidics Station 450. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (GeneChip® Scanner 3000). The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software (37). RNA was reverse transcribed with Superscript III (Invitrogen) using 1 μg of total RNA and 100 ng of random primers. Primer sequences and corresponding PCR conditions are shown in the supplemental data (Table S1). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase, used as a housekeeping gene. We used a glyceraldehyde-3-phosphate dehydrogenase-specific fragment to verify the integrity of the RNA preparation. The intensity of the amplified bands was quantified by densitometry and referred to that obtained with glyceraldehyde-3-phosphate dehydrogenase (Quantity One; Bio-Rad).

Ontology Assessment—We subjected the list of differentially expressed transcripts to gene ontology using DAVID (Data base for Annotation, Visualization and Integrated Discovery), Web-based applications that allow access to a relational data base of functional annotation (38).

Statistical Analysis—Densitometry analysis data are given as mean $\pm$ S.D. Differences were assessed by t test, and a p value less than 0.05 was considered to be significant.

RESULTS

Cell Cycle Analysis—To analyze the effects of PV serum on cell cycle progression on keratinocytes, we have incubated synchronized HaCat cells with control serum (30%) and PV serum (30%) for 24 h (Fig. 1, *a* and *b*). Treatment of HaCat cells with PV serum increased the proportion of cells in S phase (from 44 to 72%) (Fig. 1, *a*–*c*). Almost no cells (0–0.03%) were found in G_2/M phase with NS treatment, whereas a small percentage of cells was observed with PV serum treatment (4.2%) (Fig. 1, *a* and *b*).
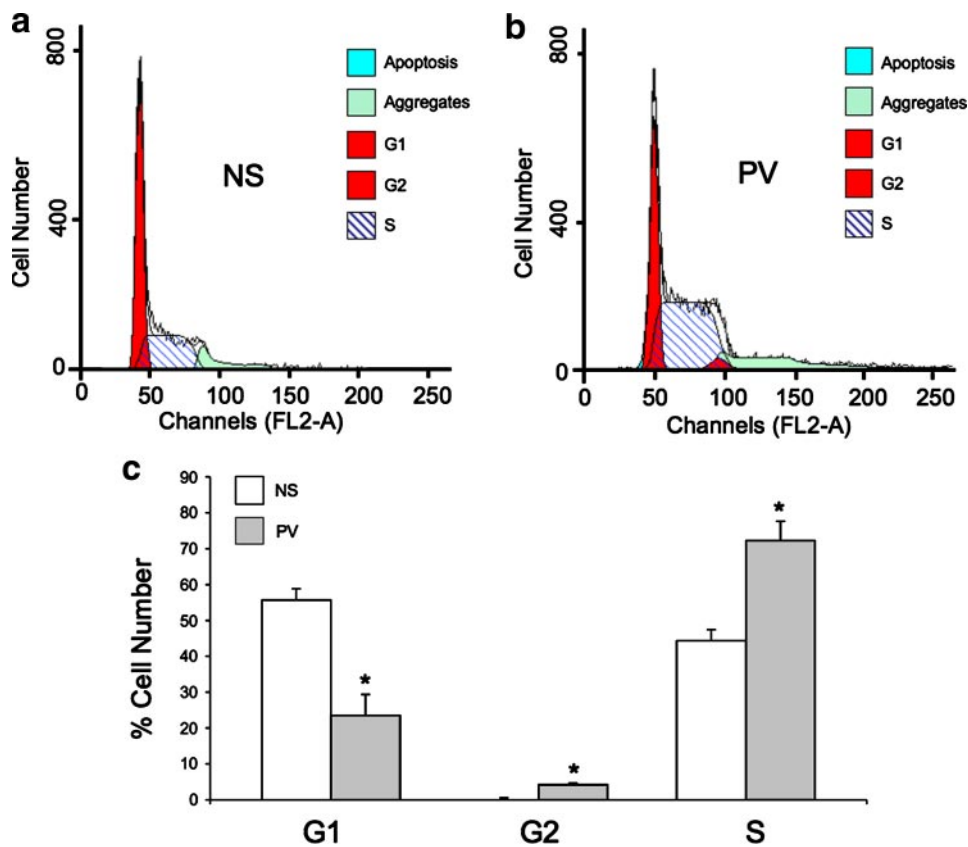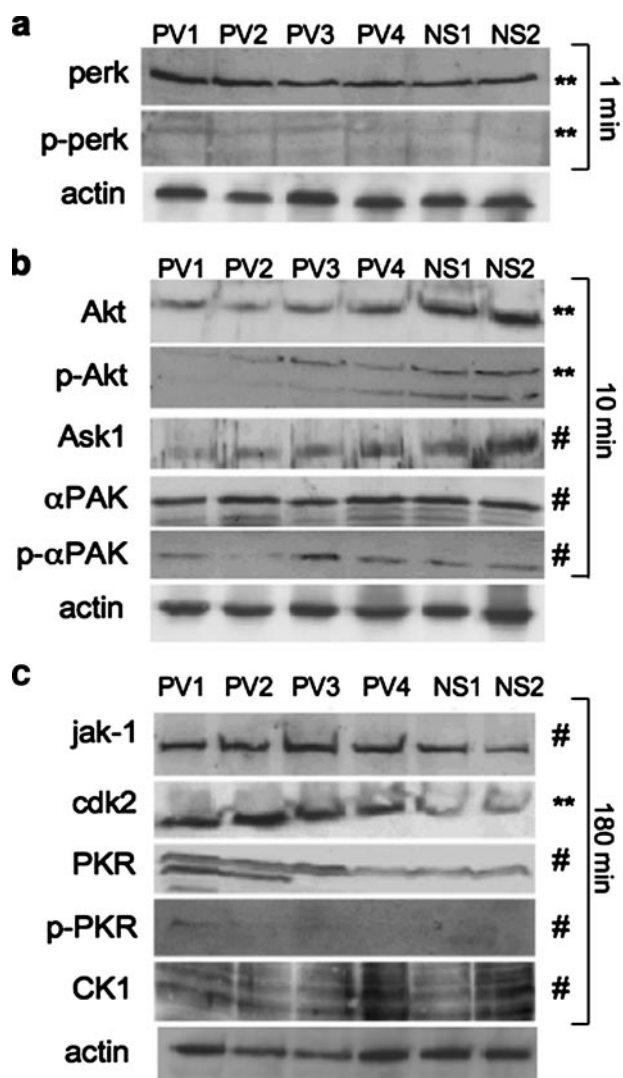
FIGURE 1. **Sera of PV patients induce changes in cell cycle progression.** Shown is fluorescence-activated cell sorting analysis of propidium iodide (*PI*) staining of starved keratinocytes treated with control serum (30%) (*a*) and PV serum (30%) (*b*) for 24 h. Cells were stained with propidium iodide and subjected to a single-color flow cytometric analysis of DNA content by red fluorescence (FL2) on a FACSCalibur. *c*, cell distribution in the G_1, S, and G_2/M phases of the cell cycle was calculated from the resulting DNA histogram with BD CellQuest software. Results are representative of six separate experiments in duplicate. Data are expressed as mean $\pm$ S.D. *, $p \leq$ 0.01 *versus* NS.

In both conditions (10 and 30%), PV sera strongly increased the amount of Cdk2 if compared with control sera, as revealed by densitometry analysis (Fig. 3*c*). Pilot experiments conducted with PV isolated IgG (instead of serum) showed a similar pattern of Cdk2 overexpression (data not shown). To evaluate whether major changes in Cdk2 levels seen in cultured cells were also appreciable *in vivo*, we performed immunohistochemical investigations on skin biopsies from PV patients and healthy controls. Data ($n = 4$ of PV patients and healthy subjects, $n = 2$) confirmed the assumption that Cdk2 was overexpressed in the skin of PV patients (Fig. 3*d*). A positive staining on the basal cell layer was not unexpected, since basal keratinocytes are active mitotically (Fig. 3*d*, *1*). PV patient skin showed an intense staining just around the site of the acantholytic cleft (Fig. 3*d*, *3* and *4*). Furthermore, there was an increase of Cdk2 expression along suprabasal layers in perilesional sites (unaffected skin) (Fig. 3*d*, *2*), indicating that Cdk2 up-regulation may precede blister formation.

Silencing of Cdk2 Prevents Cell-Cell Detachment Induced by PV Sera—Keratinocyte monolayers were exposed to whole PV and control sera for 24 and 72 h, and then we tested the ability of PV sera to induce cell-cell detachment. An antibody to Dsg1 was used for immunostaining of desmosomes in HaCat monolayers. Immunofluorescence performed on cultured cells revealed that incubation with 30% PV serum induced changes in cell shape within 24 h (Fig. 4, *a* and *b*). Punctuate clusters of fluorescein isothiocyanate fluorescence on the cell surface, referred to as contact areas among keratinocytes, appeared as elongated processes that brought together shrinking cells (Fig. 4*c*). Keratinocytes appeared completely detached from one another (Fig. 4*d*). Some cellular processes extending from the keratinocyte surface were still recognizable; however, no signs of punctuate clusters were detectable (Fig. 4*d*). To evaluate if the silencing of Cdk2 prevents cell-cell detachment induced by PV sera, keratinocytes were transfected overnight with control pool siRNA and Cdk2-specific siRNAs, treated for 24 h with 30% PV serum and 30% control normal serum (Fig. 4, *e–i*). Western blot analyses revealed that the expression of Cdk2 in the cells transfected with Cdk2-specific siRNAs was reduced (Fig. 4*e*, *lanes 3* and *4*) compared with cells transfected with the nontargeting siRNA pool (Fig. 4*e*, *lanes 1* and *2*); the level of actin remained unchanged (Fig. 4*e*, *lanes 1–4*). All of the keratinocytes appeared with a normal shape when they were treated with the

PV Serum Promotes Activation of Protein Kinases in Keratinocytes—The expression of selected kinases belonging to different branches of kinome was evaluated by Western blots. We determined optimal conditions in pilot assays and then incubated cells for 1, 3, 10, 30, 60, and 180 min with PV or control sera (and then the supplemented medium was removed and replaced with normal medium). After a short term exposure to serum, Perk was found to be up-regulated by PV sera (Fig. 2*a*). Moreover, Akt and Ask1 levels diminished in PV-treated cells in comparison with controls within 10 min (Fig. 2*b*). Phosphorylation of Akt was virtually abolished in cells exposed to PV sera. Later events included Jak-1, Cdk2, and PKR overexpression (Fig. 2*c*). On the basis of these preliminary findings, we chose to investigate more in depth Cdk2 showing higher responsiveness to PV serum (Fig. 2*c*).

PV Sera Modulate Dose-dependently Cdk2 Expression—Western blot revealed that PV serum induced a strong 10-fold increase of Cdk2 Expression in Keratinocytes Compared with Normal Serum (Fig. 3, *a* and *b*). To further investigate correlations between PV serum (10 and 30%) and Cdk2 levels, we carried out extensive experiments by incubating cells with PV and normal sera at selected time points. Keratinocytes exposed to lower quantities (10%, v/v) of PV sera exhibited a less sustained up-regulation of Cdk2 levels in comparison with those incubated with higher concentrations (30%) of PV serum (Fig. 3*c*).

FIGURE 2. **Time course studies, consisting of exposure of cells to pemphigus vulgaris (PV1–PV4) sera and control normal sera (NS1 and NS2) for 1 min (a), 10 min (b), and 180 min (c).** Shown are Western blot analyses of the following kinases: Perk, phospho-Perk, Akt, p-Akt, Ask1, αPAK, phospho-αPAK, Jak-1, Cdk2, PKR, phospho-PKR, and CK1. Loading controls were obtained by using β-actin protein. Images shown represent the typical appearance of blots obtained in three independent experiments. Differences were assessed by densitometry analyses. *, $p < 0.05$; #, $p > 0.05$.
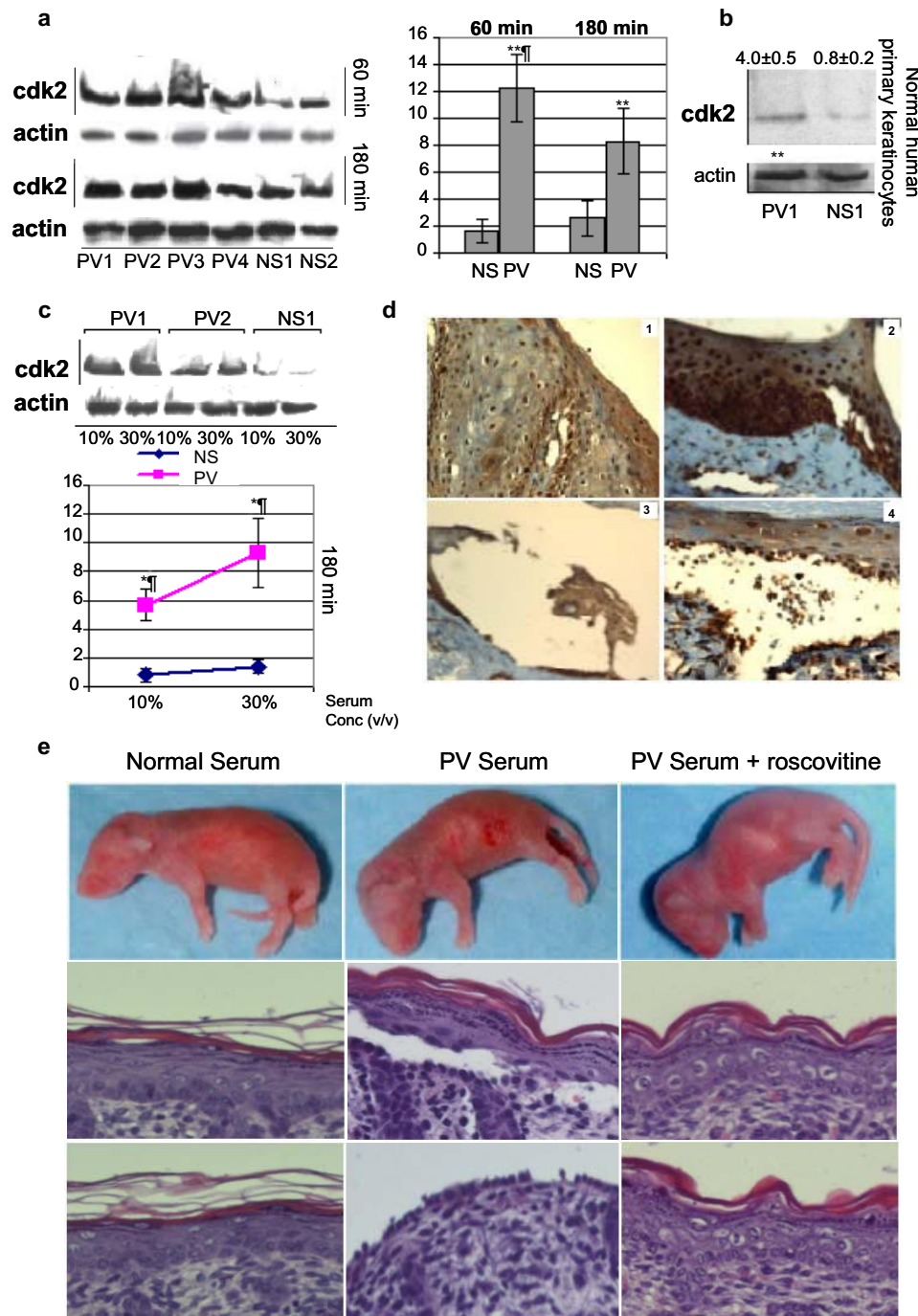
experiments, each in duplicate) for each condition (keratinocytes with normal serum, keratinocytes with PV serum) and arrays contained 14,500 well characterized human genes (22,215 total genes comprehensive of expressed sequence tag sequences) were used to measure effects of treatments of keratinocytes with PV serum *versus* normal serum. Only genes that have the threshold of 50 arbitrary detection units and absolute-fold change (FC) value greater than 1 have been included in the complete lists of genes. The FC value in a gene expression can be calculated as $FC = 2^{[Signal\ Log\ Ratio\ (SLR)]}$. The signal log ratio algorithm measures the magnitude and direction of change between transcript levels of the experimental and base-line chips. The use of logs in the analysis between the probe sets eliminates difficulties caused by one very high data point in the set masking information from lower valued data points. Base 2 is used as the log scale; therefore, a signal log ratio of 1 represents a 2-fold increase in abundance of an mRNA, and a value of -1 represents a 2-fold reduction in transcript expression (Table S2). Of course, we did not consider in those lists genes corresponding to control probes. Overall, PV serum decreased transcription of 231 genes and increased transcription of 329 genes. Important changes were observed in transcription of the genes coding for different proteins with known biological functions as follows: (*a*) components of cell organization and structure (trasgelin, keratin 13, keratin 6A); (*b*) cells adhesion molecules (laminin γ2, Rho family GTPase 3, cadherin 4, claudin 7, epithelial V-like antigen 1, protocadherin γ subfamily C3); (*c*) regulators of progression through cell cycle and apoptosis (CDC-like kinase 1, fibroblast growth factor 2, interleukin-1β, vascular endothelial growth factor B, CD70 molecule); (*d*) cell signaling proteins (follistain, Rho-guanine nucleotide exchange factor, stanniocalcin 2); (*e*) enzymes involved in general cellular metabolism (uridine phosphorylase 1, matrix metallopeptidase 14, prostaglandin E). These and other genes are listed in Table S3. To validate data obtained by microarray analysis, we performed PCR on differentially expressed genes. The levels of induction detected by reverse transcription-PCR were similar to those observed on the microarrays (data not shown). These screening experiments confirmed a previous study (39) where IgGs isolated from PV patients were used, although the use of PV serum showed a larger number of up-regulated genes (Table S2).

Evidence that Cdk2 Inhibition through Roscovitine Prevents Acantholysis in the Neonatal Mouse Model of PV—Since Cdk2 overexpression was the most remarkable phenomenon seen in the present study, 8–12-h-old neonatal BALB/c mice were injected intraperitoneally through a 30-gauge needle with 150 liters of 10-fold concentrated PV ($n = 4$) or control sera ($n = 2$). All mice receiving PV serum, but not control serum, developed skin blisters ~24 h after a single injection. The blisters resulted from suprabasilar acantholysis, as revealed by histological examination (Fig. 3*e*, *Normal Serum* and *PV Serum*). The passive transfer study was carried out in the presence of roscovitine, a well known inhibitor of Cdk2 activity (33). 100 μg of roscovitine per g of body weight dissolved in 20 μl of PBS ($n = 6$) or 20 μl of PBS as a control ($n = 6$, not shown) were administered intraperitoneally 2 h before the intraperitoneal injection of different PV sera. Remarkably, under our experimental con-

Cdk2-specific siRNA pool (Fig. 4*f*) and negative control siRNAs pool (Fig. 4*h*) in the presence of control normal serum. In the negative control, the cell detachment was more evident in comparison with Cdk-specific siRNA cells. The incubation of cells with PV in cells transfected with negative control siRNAs pool induced a significant change in cell shape (Fig. 4*g*), whereas the incubation with Cdk2-specific siRNA pool promoted a partial protection from cell-cell detachment induced by PV sera (Fig. 4*i*). Indeed, the morphometric analysis of cell-cell detachment on keratinocytes showed that the silencing of Cdk2 reduced cell-cell detachment induced by PV sera to ~8% (Fig. 4*j*). Data at 48 h were not different from those of 24 h and therefore not shown.

Modulation of Gene Expression by PV Sera in Keratinocytes Detected by DNA Microarray—The cRNA generated from a pool of three different mRNA extractions (a mean of 2 different

Modulation of Gene Expression by PV Sera in Mouse Skin Lesions Detected by DNA Microarray—To elucidate the molecular mechanism of roscovitine action on mice treated with PV serum, cRNA was generated from a pool of different mRNA isolated from skin samples of mice ($n = 6$). In this experiment, we have decided to use PV serum. In a pilot array study on keratinocytes, we determined that PV serum induced important changes in transcription of the genes coding for one and the same proteins with known biological functions involved in PV disease (39). Arrays that contained 14,000 well characterized mouse genes (22,000 total genes comprehensive of expressed sequence tag sequences) were used to measure effects of treatments of mice with PV serum *versus* normal serum, PV serum in the presence of roscovitine *versus* PV serum, and PV serum in the presence of roscovitine *versus* normal serum. In this case, only genes that have the threshold of 50 arbitrary detection units and absolute -fold change value greater or equal than 2 have been included in the complete lists of genes (Tables S4−S6). Overall, PV serum decreased transcription of 1114 genes and increased transcription of 349 genes. Roscovitine modified the gene expression profile in the presence of PV serum. Compared with the effect of PV serum alone, exposure to PV serum in presence of roscovitine showed up-regulation of 117 genes and down-regulation of 153 genes, using the same filter criteria adopted for the comparison of PV serum *versus* normal serum. Additionally, some genes down-regulated or up-regulated in the presence of PV serum alone were up-regulated or down-regulated, respectively, when treated with roscovitine (*i.e.* plakophilin 3, jagged 2, and calpain 6) (Table S7, Fig. 5, and Tables S4−S6, respectively). Importantly, the comparison of PV serum in the presence of roscovitine with NS showed that the vast majority of genes differentially expressed in PV serum alone and in presence of roscovitine were not differently expressed. In addition, Table S7 shows a list of some genes
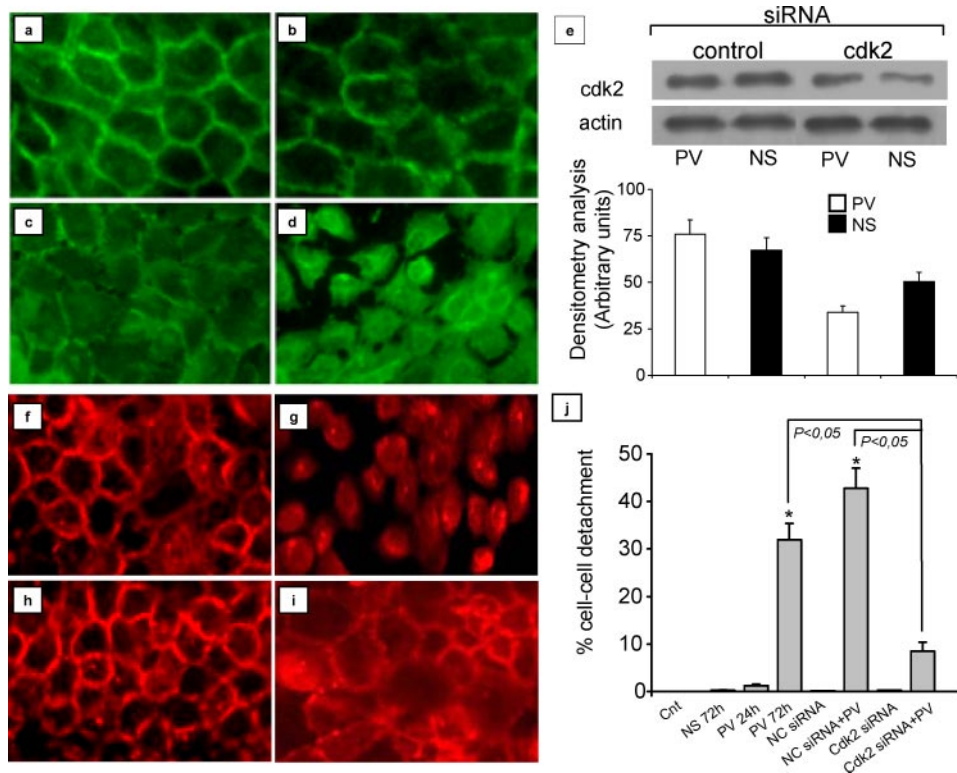
FIGURE 3. **Central role for Cdk2 in PV.** Western blotting reveals a significant increase in Cdk2 expression in both HaCaT keratinocytes (**, $p < 0.01$; **¶, $p < 0.001$) (*a*) and primary normal human keratinocytes (**, $p < 0.01$) (*b*), when exposed to PV sera (PV1–PV4), compared with normal serum (NS1 and NS2). Cdk2 overexpression was demonstrated to be a dose-dependent phenomenon, as shown by incubation of HaCaT cells with either 10 or 30% sera for 180 min (*¶, $p < 0.005$) (*c*). Immunohistochemistry analysis displayed intense staining in PV skin (*d, 2*) when compared with healthy tissues (*d, 1*). Marked cdk2 labeling was revealed throughout the basal and suprabasal layers in apparently unaffected skin as well as along the floor and the periphery of the blister (*d, 3; d, 4*). In neonatal mice injected with PV sera, pretreatment with roscovitine (100 μg/g body weight) prevents blister formation both clinically and histologically. Note that skin biopsies were done in duplicate for each mouse treated (*e*). Densitometry values (histograms) were normalized *versus* β-actin protein (equal to 10) as a loading control. Western blotting was representative of three independent experiments.

ditions, none of the mice ($n = 6$) pretreated with roscovitine developed any skin lesion, both clinically and histologically (Fig. 3*e*, *PV Serum + roscovitine*) within 24 h after intraperitoneal injection with PV sera ($n = 4$).

FIGURE 4. **Lack of Cdk2 prevents cell-cell detachment induced by PV sera.** As revealed by immunofluorescence microscopy, cells incubated with control sera did not show any morphological alteration or rearrangement of Dsg1 within 72 h after treatment (*a* and *b*). Conversely, the first changes in cell shape became evident within 24 h after treatment with PV sera, as revealed by the formation of prickle-like processes associated with cell shrinkage (*c*). 72 h after incubation with PV sera, cells were clearly detached from one another and rounded up (*d*). Effect of Cdk2-specific siRNAs on HaCaT cell-cell detachment induced by PV sera (*e–j*). Cdk2-directed siRNA inhibits Cdk2 expression, as revealed by mean densitometry analysis of different Western blots corrected by β-actin (*e*). The Cdk2-directed siRNA pool (*h* and *i*) and negative control siRNA pool (*f* and *g*) were transfected and treated with PV (*g–i*) and control normal serum (*f– h*) for 24 h before immunofluorescence analysis with the Dsg1 antibody. For the percentage of cell-cell detachment, 10 fields of different photograms were used to calculate the mean values by morphometric analysis (*j*).

regulated and up-regulated. The changes were observed in transcription of the genes coding for proteins involved in the same biological functions, such as cell adhesion, cytoskeleton organization and biogenesis, and regulation of progression through the cell cycle.

DISCUSSION

The major finding of the present study is that kinase machinery alterations occurring in PV acantholysis crucially involve Cdk2. This evidence was obtained both in cultured cells and *in vivo*, by using skin biopsies of PV patients. We observed also that PV serum increased the cell cycle progression. Second, we revealed that silencing of Cdk2 was able to reduce disruption of cell-cell contacts in keratinocytes exposed to PV serum. Hence, we assumed that Cdk2 is essential for PV acantholysis *in vitro*. Third, we reported that pharmacological inhibition of Cdk2 by roscovitine prevented blister formation in the neonatal mouse model of the disease. Thus, Cdk2 is essential for PV blistering *in vivo*. Fourth, we showed by microarray analysis that PV sera induced multiple changes in gene expression, and regulatory events involve both kinases and several adhesion molecules. Our complete set of experiments also showed that some kinases are involved in PV-induced acantholysis.

down- and up-regulated in presence of PV serum and reestablished expression of genes by roscovitine. The biological process ontology and KEGG pathway terms associated with the differentially expressed genes were examined using the online available DAVID bioinformatics data base. The reciprocal changes were observed in transcription of the genes coding for proteins with known biological functions as follows: (*a*) components of cell organization and structure (keratins, transglutaminase, and involucrin); (*b*) cell adhesion molecules (jagged 2, desmoplakin, bystin-like, reelin, and calpain 6); (*c*) regulators of cell cycle progression (cyclin-dependent kinase 8, cyclin D1, cyclin D2, and cyclin G associated kinase; (*d*) cell death (cyclin-dependent kinase inhibitor 1A (P21), Fas death domain-associated protein, and growth arrest-specific 1); (*e*) defense response (CD93 antigen and histocompatibility 2, D region); (*f*) immunoresponse (histocompatibility 2, D region locus 1, and interferon-activated gene 203). As usual, to validate data obtained by microarray analysis, we performed PCR on several differentially expressed genes involved in cell communication pathways. The levels of induction detected by reverse transcription-PCR were similar to those observed on the microarrays (Fig. 5).

Shared Microarray Results—Comparing results from both human and mice microarrays, different genes were down-

Cdk2 overexpression was demonstrated in suprabasal layers of perilesional (unaffected) skin, indicating that PV-induced changes in Cdk2 levels may precede the phenotypic manifestations of disease. The sustained overexpression of Cdk2 (12-fold increase) led us to investigate whether this key cell cycle regulator was causally related to PV acantholysis. Thus, we knocked out Cdk2 from keratinocytes through silencing techniques and then tested the ability of PV sera to induce adhesion loss in such a Cdk2-deficient cell line. Interestingly, the absence of Cdk2 conferred resistance to the action of PV serum, since keratinocytes lacking Cdk2 did not develop acantholysis *in vitro*. In previous studies, Caldelari *et al.* (40) used a similar targeted approach and reported that plakoglobin knock-out keratinocytes were unresponsive to PV IgG. However, they established PG$^{-/-}$ cell cultures from mouse skin. Thus, our study is the first working silencing gene approach to study the pathophysiology of PV in human keratinocytes. Here, we have exposed cultured cells to PV patient sera, as proposed by Swanson and Dahl (23). On the basis of both our preliminary experiments with PV IgG and other studies (22–25, 28), we preferred to avoid "nonphysiological" experimental conditions and thus set
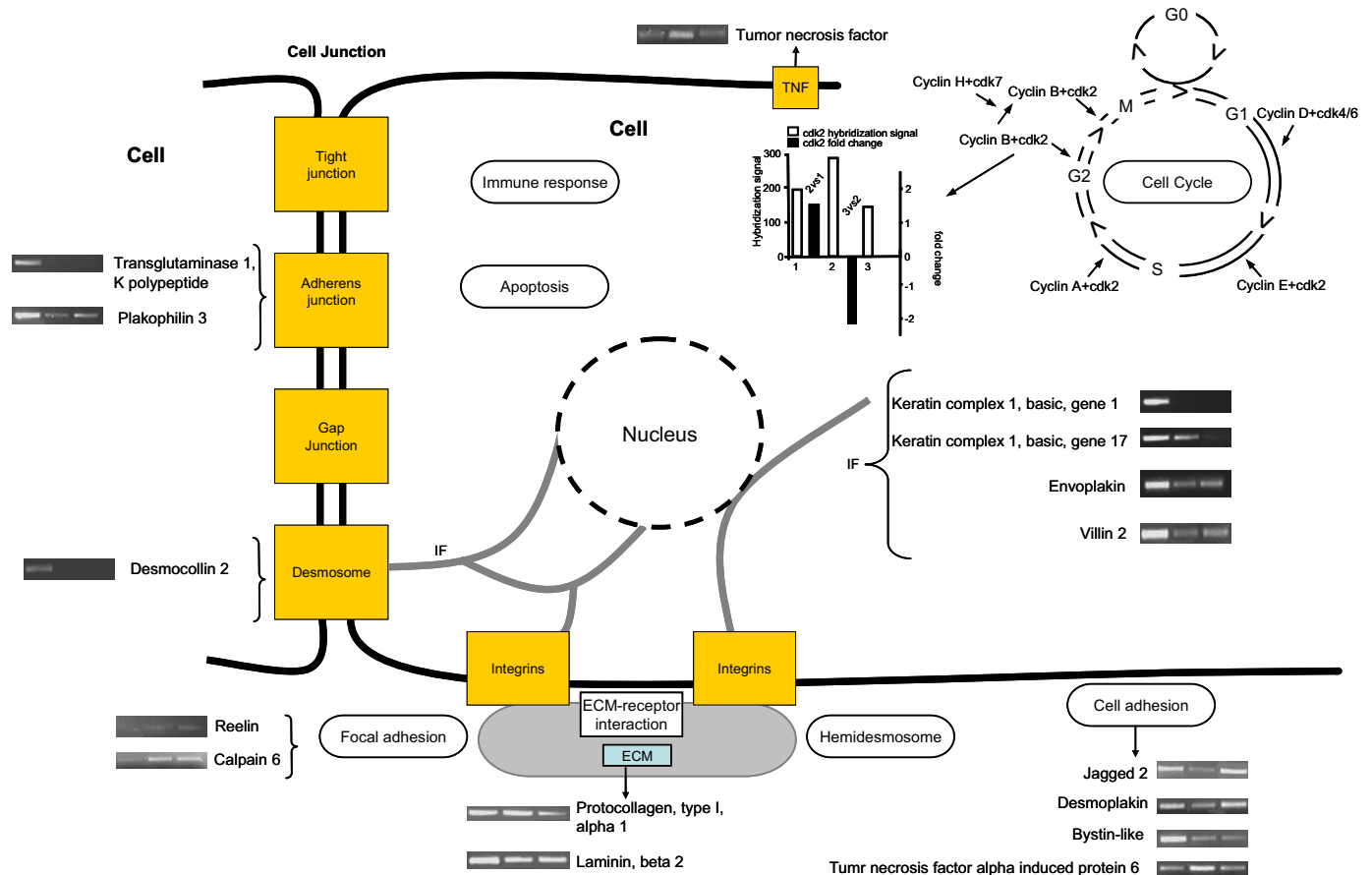
FIGURE 5. **Pathway diagram (DAVID/KEGG data base) showing genes involved in cell communication differentially regulated by PV serum with and without roscovitine treatment.** The genes showed were confirmed by reverse transcription-PCR. For each amplification, the *first lane* on the *left* is normal serum, the *second lane* is PV serum, and *third lane* on the *right* is PV serum with roscovitine treatment. PV serum induced a significant variation in gene expression implicated in direct cell-cell contacts. Roscovitine treatment partially reestablished the expression of these genes. Emphasis is put on Cdk2-dependent cell cycle alterations and respective expression signals by microarray analysis (-fold change and hybridization signals; *1*, NS; *2*, PV serum; *3*, PV serum + roscoivitine).

the complete panel of experiments in the presence of PV serum instead of isolated IgG. A mouse injected with roscovitine, an inhibitor of Cdk2, before receiving PV serum did not develop any skin lesion. This finding provided strong evidence that activation of Cdk2-mediated signaling is a crucial event of PV acantholytic process *in vivo* and that pharmacological block of Cdk2 can prevent PV skin blistering in mice. Recent studies report that also p38 mitogen-activated protein kinase and c-Myc may be pharmacologically targeted in order to inhibit PV-like blistering (12, 13, 41). We do not actually know whether PG, p38 mitogen-activated protein kinase, Cdk2, and c-Myc are members of the same "acantholytic cascade" or whether they represent effectors of different signal transduction leading independently to cell-cell detachment. However, results reported here reveal Cdk2 to be a key protein in PV pathogenesis and open new perspectives toward a more targeted therapy of the disease. Indeed, the current treatment of PV patients is based on the nonselective use of steroidal and immunosuppressive agents (2). Our findings implicate that the molecular effects of PV could be targeted by using selective Cdk2 inhibitors. However, the safety of such inhibitors in humans must be properly addressed. Among novel therapeutic approaches, adjuvant B-cell depletion by rituximab is effective in otherwise therapy-resistant bullous autoimmune disorders, including PV, but may be associated with substantial adverse effects, including fatal outcomes (42). PV blistering is a complex phenomenon that parallels the disruption of desmosomal structures. Although it has become clear that acantholysis is not just the result of a direct steric hindrance between IgG and Dsg3, it is not yet clear how PV serum affects the regulation of cell adhesion strength in keratinocytes. Indeed, although phosphorylation events occur to rapidly regulate protein activity, changes in gene expression take place more slowly, but they are expression of a stable response of the cell to the external clues. Here, in order to define disease mechanisms, we took an empirical unbiased approach analyzing keratinocytes and mice skin treated with PV by large genome microarray technologies. Our data indicate a deregulation of transcripts primarily involved in cell organization and structure of epithelial tissue and keratins.

Different genes down-regulated in PV serum-treated keratinocytes were also down-regulated in another study wherein isolated IgGs from PV patients were used (39). It is noteworthy that the use of PV serum showed a larger number of up-regulated genes. The microarray that we designed on mouse skin treated with PV serum was intended to evaluate the expression

of known and unknown genes by comparing the gene expression with the response to roscovitine.

Keratins are major structural proteins in epithelial cells (43–45). They make up the largest subgroup of intermediate filament proteins and form a dynamic network of 10–12-nm filaments, built type I/type II heterodimers, in the cytoplasm of epithelial cells (43–45). A major function of keratin is to protect epithelial cells from mechanical and nonmechanical stresses that cause cell rupture and death (43–45). We have found several genes encoding components of intracellular desmosome junction. Desmosomes together with adherens junction represent the major adherens cell-cell junctions of epithelial cells. Both types of junctions are connected with the cytoskeleton and represent sites of mechanical coupling between cells. In contrast to adherens junctions, which are linked to the actin microfilament system, desmosomes are associated with intermediate filaments. Moreover, we have found that PV serum induced down-regulation of different desmosomal cadherins, such as desmocollin 2 and desmoglein 2 (46). Keratinocytes grown in low Ca^{2+} medium proliferate but do not form cell-cell contacts. A rise in Ca^{2+} concentration induces rapid assembly of adherens junctions and desmosomes. Variations in Ca^{2+} concentration seem to interfere with normal desmosome assembly. Therefore, changes in intracellular Ca^{2+} concentration may induce regulatory signal (46). This putative role of Ca^{2+} in keratinocyte signaling leading to acantholysis is consistent with the finding that PV autoimmunity targets acetylcholine receptors (47). In fact, PV patients develop autoantibodies against the $\alpha9$ acetylcholine receptor Ca^{2+} channel with dual muscarinic and nicitic activity, which regulates epidermal adhesion (48, 49). Interestingly, we found in the present study an up-regulation (calcium channel, voltage-dependent, L type, $\alpha1D$ subunit) and down-regulation (gap junction membrane channel protein $\alpha1$) of genes involved in calcium transport. In addition, cell signaling induced by the nondesmoglein autoantibodies produced by PV patients that may target keratinocyte acetylcholine receptors modulates Src and EGF receptor kinase, which have been shown to play an important role in regulation of intracellular adhesion (50). Moreover, we have found recently that PV IgG can recognize an antigen other than desmoglein 3 on peripheral blood mononuclear cell surface (25). Interestingly, we found an up-regulation of inflammatory cytokines previously reported to be involved in PV acantholysis, such as TNF and interleukin-10 (11, 51). Thus, PV serum seems to reduce the adhesion strength among keratinocytes by acting on the transcriptional level through the down-regulation of desmosomal proteins. It is noteworthy that these "slower" changes in gene regulation were abrogated by roscovitine, thus showing that Cdk2 is crucial in orchestrating intracellular signaling, which impairs desmosome adhesion strength in pemphigus, thus resulting in acantholysis. To date, further experiments are being undertaken in our laboratories to further assess the precise regulatory framework of cluster genes involved in acantholysis during PV. Finally, it has been long believed that Cdk2 binds to cyclin E or cyclin A and exclusively promotes the G_1/S phase transition and that Cdc2-cyclin B complexes play a major role in mitosis. There is now evidence that Cdc2 binds to cyclin E (in addition to cyclin A and B) and is able to promote the G_1/S transition (reviewed in Ref. 29). This novel concept indicates that Cdk2 and/or Cdc2 can drive cells through G_1/S phase in parallel. Thus, a new pathogenic scenario for Cdk2-dependent alterations in acantholysis present PV and other diseases should be investigated.

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Evidence of Key Role of Cdk2 Overexpression in Pemphigus Vulgaris

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