

α II-Spectrin Is Critical for Cell Adhesion and Cell Cycle*

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Spectrins are ubiquitous scaffolding components of the membrane skeleton that organize and stabilize microdomains on both the plasma membrane and the intracellular organelles. By way of their numerous interactions with diverse protein families, they are implicated in various cellular functions. Using small interfering RNA strategy in the WM-266 cell line derived from human melanoma, we found that α II-spectrin deficiency is associated with a defect in cell proliferation, which is related to a cell cycle arrest at the G₁ phase (first gap phase), as evaluated by DNA analysis and Rb phosphorylation. These observations coincided with elevated expression of the cyclin-dependent kinase inhibitor, p21^{Cip}. Concomitantly, spectrin loss impaired cell adhesion and spreading. These cell adhesion defects were associated with modifications of the actin cytoskeleton, such as loss of stress fibers, alterations of focal adhesions, and modified expression of some integrins. Our results provide novel insights into spectrin functions by demonstrating the involvement of α II-spectrin in cell cycle regulation and actin organization.

First identified at the intracellular surface of the erythrocyte plasma membrane, the spectrin-based skeleton is considered as a nearly ubiquitous and complex spectrin-actin network in metazoan cells (1).

Spectrins are giant extended flexible molecules composed of two subunits (α and β) that intertwine to form $\alpha\beta$ heterodimers. Spectrin is normally considered to exist as tetramers resulting from self-association of $\alpha\beta$ dimers. Spectrin tetramers constitute the filaments of the lattice, the nodes of which are cross-linked by actin filaments. This spectrin-based skeleton is bound to various transmembrane proteins either directly, or more frequently through two connecting proteins, ankyrin and protein 4.1. In mammals, the spectrin family currently includes seven genes encoding for two α -subunits (α I and α II), four "conventional" β -subunits (β I to β IV) and one β heavy subunit (β V), as well as multiple alternatively spliced variants, each of these species presenting its specific cellular expression pattern. For example, whereas α I-spectrin is essentially expressed in the mature erythrocyte, α II-spectrin is the most common form in nucleated cells.

The functions clearly determined up to date for the spectrin network emerge from human mutations associated with dis-

eases as well as from animal models. Numerous studies on red cells, particularly those in hereditary hemolytic anemia, have clearly established its importance for supporting cell shape and for maintaining cell membrane integrity and stability (2, 3). In nucleated cells, the spectrin-based skeleton has been shown to participate in the stabilization or activation of several specialized membrane proteins, as recently reported for the TRCP channels (4). The direct interaction between TRCP4 channel and spectrin is involved in the regulation of the channel surface expression and activation. One consistent feature observed when spectrin or its binding partner ankyrin are lost or defective, is a failure of interacting membrane proteins to accumulate at the appropriate site. The loss of β IV-spectrin observed in quivering mice with hearing defect is associated with a mislocation of voltage-gated channels from the axon initial segment and the node of Ranvier (5, 6). In humans, β III-spectrin mutations, which are responsible for spinocerebellar ataxia type 5, are associated with a mislocation of the glutamate transporter EAAT4 at the surface of the plasma membrane (7, 8). In *Drosophila*, loss of β -spectrin led to loss of Na,K-ATPase from the basolateral domain of epithelial cells (9). In an extreme case, loss of a variant of β II-spectrin in mice led to death *in utero* (10).

Although the consequences of loss of function of β -spectrins and ankyrins are progressively better explained, the cellular consequences of α -spectrin defects are less well established, except in the context of red blood cells: mutations in the gene coding for α I-spectrin mainly expressed in mature red blood cells from mammals, are associated with severe hemolytic anemia and in some cases with a short survival (11, 12). Defects in the unique α -spectrin ortholog to the α II-spectrin of vertebrates are lethal in *Drosophila melanogaster* and *Caenorhabditis elegans* larvae, arguing for the crucial role of this protein (13–15). Up to now, there are no mammalian models describing α II-spectrin knock-out. In this paper, we studied the relative contributions of α II-spectrin to cell behavior using a small interfering RNA (siRNA)² knock-down approach in a cellular model. For the first time we show that depletion of α II-spectrin is associated with a loss of cell adhesion as well as with an arrest of cell proliferation.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antibodies against lamin and paxillin were purchased from Santa Cruz Biotechnology, against p15, p16, and p27 proteins were from Cell Signaling. Monoclonal antibodies against α II-spectrin, retinoblastoma

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² The abbreviations used are: siRNA, small interfering RNA; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SH3, Src homology domain 3.

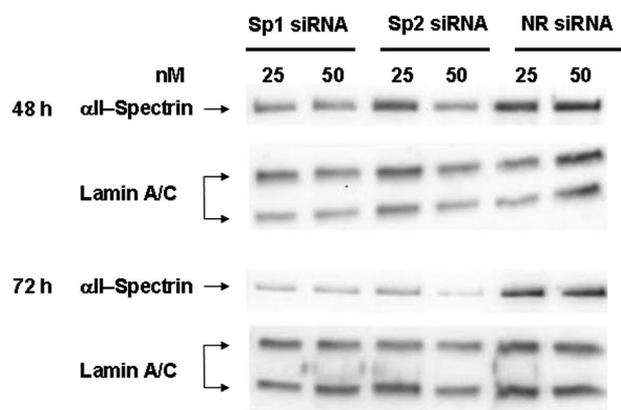


FIGURE 1. The down-regulation of α II-spectrin expression by siRNA in the WM-266 melanoma cell line is effective after 48 h transfection. WM-266 cells were transfected with either siRNAs targeting α II-spectrin (*Sp1 siRNA* and *Sp2 siRNA*) or non-relevant control siRNA (*NR siRNA*) at two concentrations (25 and 50 nM). The SDS cell lysates obtained at 48 and 72 h after transfection were analyzed by Western blotting with antibodies directed against α II-spectrin and lamin A/C (used as a loading control). Spectrin depletion is obvious at 48 h, but more pronounced at 72 h.

protein (Rb), integrins (α 4, α 5, α 6, α V, β 3, and β 4), p16 and p21 proteins were purchased from BD Biosciences. Monoclonal antibodies against α 3, β 1, β 3, and α V β 3 integrins were purchased from Chemicon International. Rabbit polyclonal antibodies against α II-spectrin were previously described (16).

Cell Culture and Transfection—The human melanoma cell line, WM-266-4 (derived from a metastatic site of a malignant melanoma) (ATCC, CRL-1676), was grown at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine or glutamax, 1 mM sodium pyruvate and penicillin/streptomycin. Transfections were performed with siRNA duplexes at different concentrations using jetSI-ENDO (PolyPlus) according to the manufacturer's procedure: transfection reagent dilution (1 μ l into 25 μ l of serum free Opti-MEM) was mixed to siRNA duplexes diluted in Opti-MEM (25 μ l). After 30 min incubation, the mixture (1 volume) was added to 5 volumes of cells in suspension (100,000 cells/ml).

The transfection efficiency was estimated at 24 h after transfection by flow cytometry (FACSCalibur flow cytometer, BD Biosciences) using either negative control siRNA labeled with Alexa Fluor 488 (Qiagen) or Silencer FAM-labeled GAPDH siRNA (Ambion). Cell viability was also estimated by flow cytometry after cell treatment with 5 μ g/ml propidium iodide. Six siRNA duplexes targeting human α II-spectrin were tested: four were from Dharmacon (individual siGENOME duplex D-009933-01, D-009933-02, D-009933-03, and D-009933-04), two were from Ambion (Silencer Pre-designed siRNAs 12798 and 142727); negative silencer control siRNAs (non-relevant) and siRNA targeting p21 were from Dharmacon (siCONTROL™ Non-Targeting siRNA Pool and siGENOME SMART pool M-003471, respectively).

Western Blot Analysis—After two washes with prewarmed Dulbecco's PBS (Invitrogen), the cells were directly lysed on plates in phosphate-buffered saline containing 1% SDS and anti-protease mixture (Sigma). Protein concentrations were estimated in a colorimetric assay using the BCA method (microAssay Uptima), using bovine serum albumin (BSA) as a standard protein. Aliquots of cell lysates containing equal amounts of proteins (between 8 and 20 μ g) were resolved on SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (either 0.45 or 0.1 μ m).

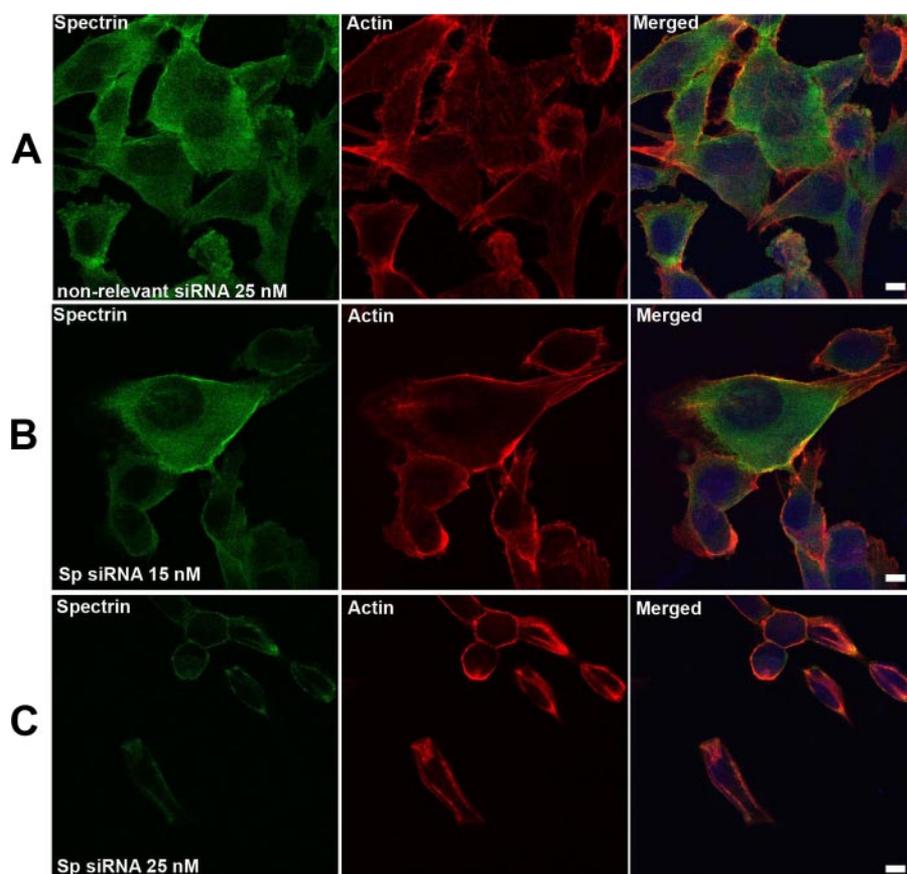


FIGURE 2. α II-Spectrin siRNA knockdown is associated with modifications of cell shape and spreading as revealed by immunofluorescence analysis. WM-266 cells were cultured for 72 h following transfection with non-relevant siRNA at 25 nM (A) or siRNA 1 targeting α II-spectrin at 15 (B) and 25 nM (C). The non-relevant siRNA-treated cells (A) are spread with the presence of actin stress fibers (labeled with phalloidin, pseudo-colored in red) and α II-spectrin (stained with polyclonal anti-Sp- α II antibodies, pseudo-colored in green) has a diffuse distribution throughout the cytoplasm, and is also associated with the plasma membrane. After siRNA knockdown of α II-spectrin expression, transfected cells exhibited a concomitant decrease in spectrin labeling, cell size, and spreading, together with a reduction of the F-actin stress fibers network (B and C). The merged image in panel B is particularly illustrating of the cellular phenotype modifications, as indicated by the presence of a cell with normal spectrin labeling. Nuclei were counterstained with TO-PRO 3 (pseudo-colored in blue). Bar, 10 μ m.

(Protan, Schleicher & Schuell) using a Tris glycine buffer (in the presence of 10% methanol for 0.1- μ m membrane). After saturation in 5% nonfat milk, 0.05% Tween 20, PBS buffer, pH 7.5, the membranes were then probed overnight at 4 °C with the indicated primary antibodies. After extensive washing, blots were incubated for 1 h at room temperature with secondary

antibodies conjugated with horseradish peroxidase (Nordic Immunological Laboratories). Immune complexes were detected using the SuperSignal West Pico chemiluminescence substrate (Pierce). The chemiluminescence was quantified using the Quantity One® One-dimensional Analysis software after acquisition with the Molecular Imager® Gel Doc™ (Bio-Rad).

Cell Cycle Analysis and Apoptosis Study by Flow Cytometry—The cell cycle was analyzed by estimation of the DNA content by flow cytometry. The WM-266 cells were detached by trypsin-EDTA treatment (Invitrogen). After washing, 5×10^5 cells were first fixed with 1% of paraformaldehyde on ice for 15 min, then after two washes in PBS, with 70% ethanol for at least 30 min. The DNA was stained with 50 μ g/ml propidium iodide concomitantly with RNase treatment (0.2 mg/ml in PBS, 0.2% BSA for 30 min in the dark).

Apoptosis was studied using DiOC₆, a green fluorescent cationic dye that accumulates in active mitochondria and is used to follow changes in the membrane potential of mitochondria that occur during apoptosis. Transfected cells were detached by trypsin-EDTA. After washing in PBS, 5×10^5 cells were stained with 80 nM DiOC₆ in PBS, 0.2% BSA and dead cells were detected by staining with 15 μ g/ml propidium iodide.

Immunofluorescence Studies—Cells grown on CC2 slides (Nunc) were washed in prewarmed Dulbecco's PBS, fixed in 4%

paraformaldehyde, permeabilized with 0.5% Triton X-100, and saturated 30 min either with buffered saline solution containing 0.1% BSA or with Image-iT™ Signal Enhancer (Molecular Probes). Primary and secondary antibody dilutions were made either in buffered saline solution containing 0.1% BSA or in background reducing buffer (DakoCytomation). When indicated, nuclei were counterstained with TO-PRO 3 (Molecular Probes) after treatment with RNase for 30 min. In a final step, immunolabeled cells were mounted in ProLong™ Antifade Gold solution. Secondary labeled anti-IgG antibodies were purchased from Molecular Probes (Alexa Fluor™ 488 and Alexa Fluor™ 568). F-actin was labeled with Alexa Fluor 568-phalloidin (Molecular Probes). The fluorescence was observed by confocal microscopy using either a Zeiss LSM 510 META or a Nikon Eclipse TE300 microscope up-graded with a D-Eclipse C1 confocal system.

Study of Integrin Expression by Flow Cytometry—The surface expression of integrins (α 3, α 4, α 5, α V β 3, β 1, and β 3 integrins) was studied 72 h after transfection. The cells were

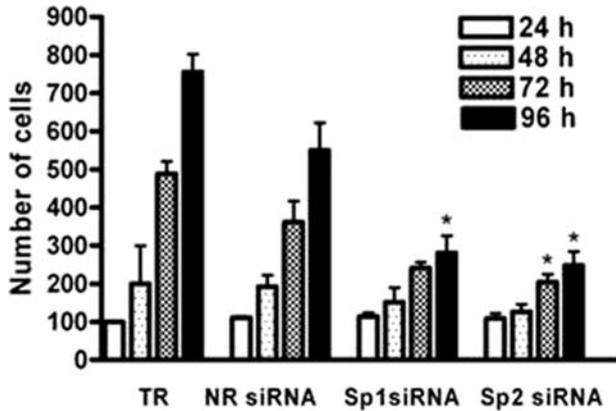


FIGURE 3. Spectrin depletion is associated with a defect in cell proliferation. Cell growth was evaluated during 4 days by cell counting in samples treated with transfection reagent (TR), siRNA targeting all-spectrin (Sp1 and Sp2), or non-relevant siRNA (NR) at 25 nM. The number of cells in samples treated with spectrin siRNAs was significantly decreased (*, $p < 0.05$) compared with samples treated with NR siRNA after 72 and 96 h treatment. These data were obtained from three separate transfections.

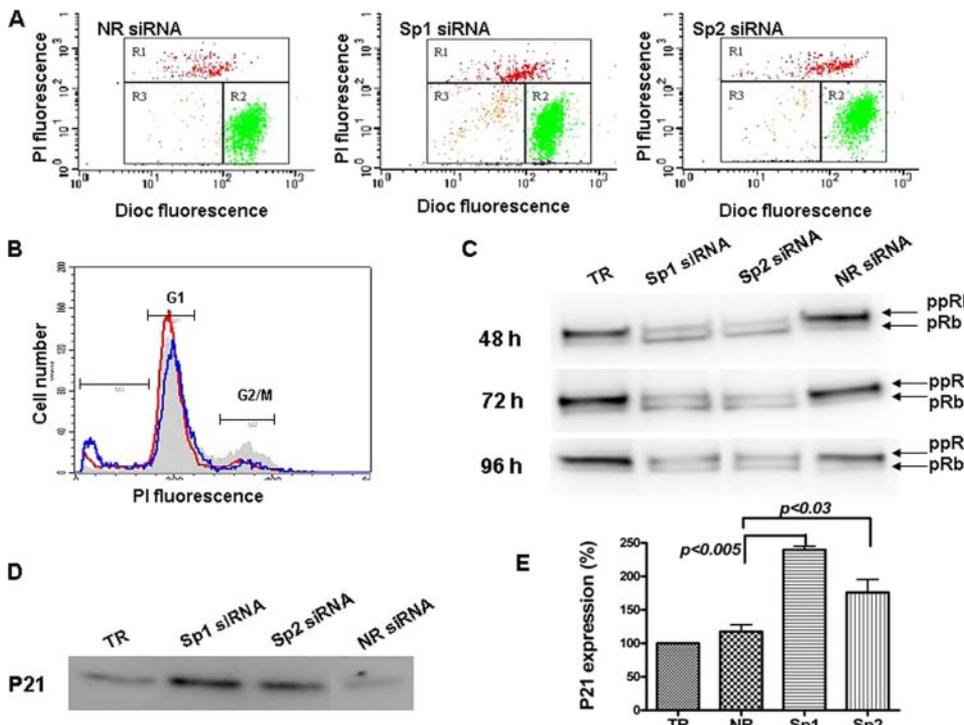


FIGURE 4. Spectrin depletion inhibits cell proliferation via G₁ arrest. *A*, spectrin depletion was not obviously associated (at 72 h) with apoptosis as evaluated by the presence of a few cells without DiOC₆ labeling (R3) and with cell death as evaluated by propidium iodide (PI) labeling (R1). *B*, the number of cells in the G₂/M and S phases (as evaluated by DNA labeling with PI) was decreased in samples treated with Sp1 siRNA (red line) and Sp2 siRNA (blue line) as compared with sample treated with non-relevant siRNA (gray background), indicating a cell cycle arrest at the G₁ phase. The peak at the left of the curve corresponds to cell fragments (not included in this analysis). *C*, the retinoblastoma Rb protein was mainly in a hypophosphorylated status in cells treated with spectrin siRNA (Sp1 and Sp2) when this protein was essentially highly phosphorylated in cells treated with either transfection reagent (TR) or non-relevant siRNA (NR), confirming an arrest of the cell cycle at G₁. *D*, the Western blot probed with antibodies directed against the CDK inhibitor p21 revealed an increased expression of p21 in samples treated with spectrin siRNAs. This increased expression was highly significant as shown in panel *E*.

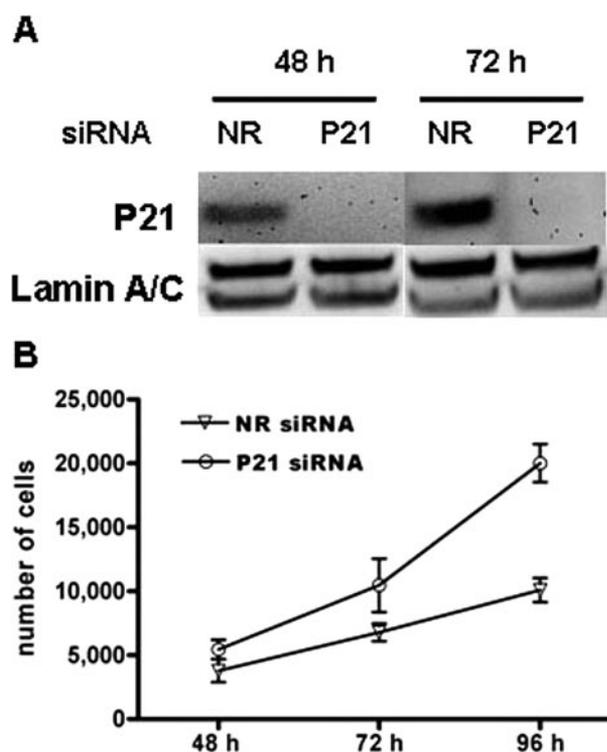


FIGURE 5. Protein p21 acts as a negative regulator of cell proliferation in the WM-266-4 cell line. Cells were transfected with siRNAs either targeting p21 or non-relevant control (*NR siRNA*) at 30 nM. The SDS cell lysates obtained at 48 and 72 h after transfection were analyzed by Western blotting with antibodies directed against p21 and lamin A/C (used as a loading control). Protein p21 was no longer detected at 48 and 72 h (A). The number of cells in samples treated with p21 siRNAs was significantly increased as compared with samples treated with NR siRNAs ($p < 0.05$) (B). These data were obtained from two separate transfections.

detached using Versene. After washing, 5×10^5 cells were diluted in 100 μ l of ice-cold PBS, 0.2% BSA and incubated 90 min at 4 °C with primary monoclonal antibodies diluted at 5 μ g/ml in PBS, 0.2% BSA. After two washes with ice-cold PBS, the cells were incubated 45 min at room temperature with Alexa 488-conjugated goat anti-mouse IgG diluted at 5 μ g/ml in PBS, 0.2% BSA. Cells were then washed and suspended in 500 μ l of PBS, 0.2% BSA containing 0.2 μ g/ml propidium iodide to exclude dead cells from flow cytometric analysis. The background was assessed by cell labeling with an antibody directed against V-CAM, an adhesion molecule not expressed in WM-266 cells. Quantifications were done using the QIFIKIT® beads kit from DAKO Cytomation.

Static Cell Adhesion Assays—Adhesion assays were performed at 72 h after transfection on culture dishes. Control and transfected cells were detached using trypsin-EDTA. After washing in Dulbecco's PBS, the transfected cells were stained with 10 μ g/ml Calcein AM (Molecular Probes) when the control cells were stained with 1 μ g/ml Hoechst 33342. After two washes in complete culture medium, transfected cells were mixed with control cells as internal standard in a 1/1 ratio. These mixtures made of two cell populations in suspension were plated in triplicate on 12-well plates (2×10^6 cells per well) and incubated for 2 h at 37 °C in 5% CO₂. After two washes with complete culture medium, the remaining adherent cells were visualized by fluorescence using Evolution VF camera (Media

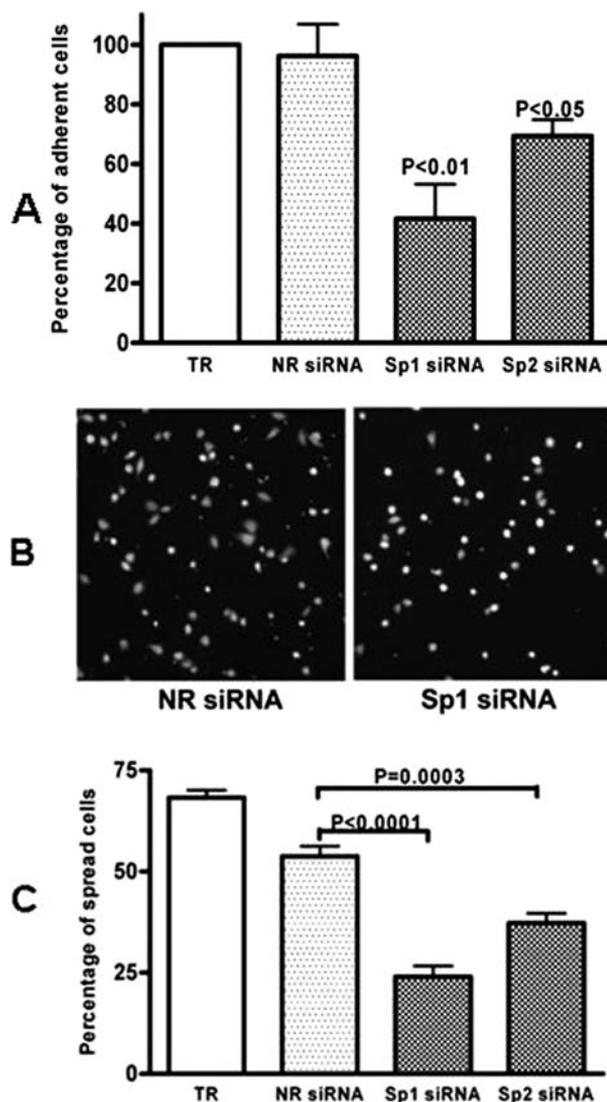


FIGURE 6. Spectrin depletion alters static adhesion and cell spreading. A, adhesion assays were performed on the WM-266-4 cells at 72 h after transfection with either siRNAs targeting α II-spectrin (*Sp1* and *Sp2*) or non-relevant siRNA (*NR siRNA*) at 30 nM or without siRNA (*TR*). Two hours after replating, the adhesion of WM-266-4 cells treated with siRNAs targeting α II-spectrin was significantly decreased when compared with cells treated with non-relevant siRNA as indicated by the number of remaining cells after washing. Panel B shows the Calcein-labeled cells, 4 h after seeding. Cells treated with Sp1 siRNA were mainly rounded and consequently brighter when most of the cells treated with NR siRNA were spread and consequently display a more diffuse fluorescence. C, according to the fluorescence intensity in Calcein-treated cells evaluated by the Image-Pro Plus software, the number of spread cells treated with Sp1 and Sp2 siRNAs were significantly decreased compared with cells treated with non-relevant siRNA (*NR siRNA*).

Cybernetics). Ten images were acquired for each sample of mixed cells, and adherent cells were counted using Image-Pro® Plus software. The results are expressed as the mean percentages of adherent transfected cells versus control adherent cells (100%). Spread cells were discriminated from round cells according to fluorescence intensity of Calcein, the round cells showing a higher intensity. To check the involvement of integrins in adhesion, cells were incubated 90 min with blocking antibodies directed against α 3, α 5, and α V β 3 integrins (15–25 μ g/ml) before seeding. Control cells were incubated with IgG isotype (25 μ g/ml).

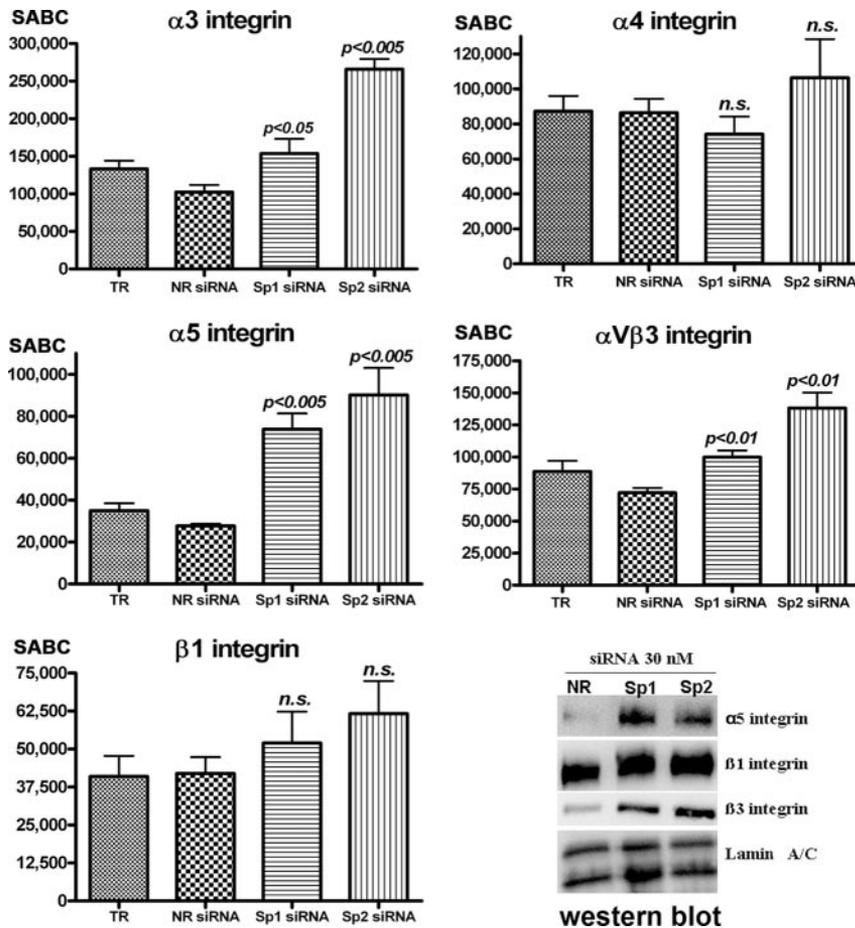


FIGURE 7. The expression of integrins is modified in cells treated with siRNA targeting α II-spectrin. The membrane expression of $\alpha 3$, $\alpha 5$, and $\alpha V\beta 3$ integrins were evaluated as specific antibody binding capacity units (SABC) by flow cytometry was significantly increased in cells transfected with Sp1 siRNA and Sp2 siRNA (at 30 nM, 72 h after transfection) when compared with the cells treated either with transfection reagent (TR) or with non-relevant siRNA (NR siRNA). The membrane expression of $\alpha 4$ and $\beta 1$ integrins was not significantly modified. The amounts of $\alpha 5$ and $\beta 3$ integrins present in 1% SDS cell lysates (15 μ g of proteins, analyzed on 7% SDS polyacrylamide gels, followed by Western blots) were significantly higher than in cells transfected with non-relevant siRNA (NR). The content of cell lysates in $\beta 1$ integrin was not significantly modified in the different samples. Lamin A/C was used as a loading control of cell lysates.

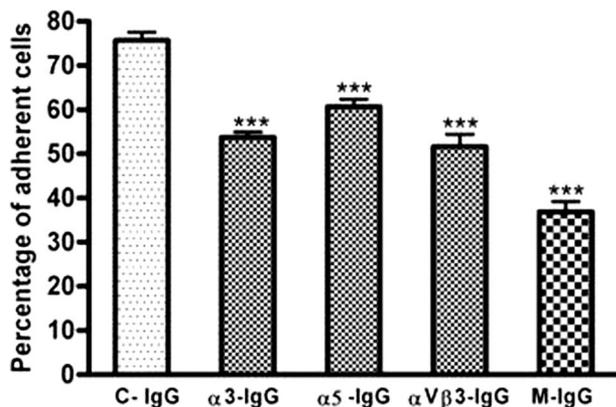


FIGURE 8. The $\alpha 3$, $\alpha 5$, and $\alpha V\beta 3$ integrins are implicated in the WM-266 cell adhesion. Prior to adhesion assays, the cells were incubated with either IgG or blocking antibodies directed against integrins ($\alpha 3$, $\alpha 5$, and $\alpha V\beta 3$ integrins) or a mixture of antibodies directed against these integrins; these cells were stained with Calcein and mixed in a 1/1 ratio with control cells labeled with Hoechst before seeding. The data are expressed as the percentage of adherent cells after treatment with antibodies (stained by Calcein) compared with the number of adherent control cells (labeled with Hoechst). Cell treatment with antibodies directed against integrins reduced cell adhesion significantly (***, $p < 0.001$).

RESULTS

The Down-regulation of α II-Spectrin Expression by siRNA in the WM-266 Melanoma Cell Line Is Effective after 48 h Transfection—

To select optimal and specific siRNAs targeting α II-spectrin, six siRNA duplexes were tested on spectrin expression in the WM-266 melanoma cells during 4 days after transfection. In a first step, the transfection conditions were optimized using non-relevant labeled siRNA to get the best transfection efficiency associated to a good level of cell viability as assayed by propidium iodide exclusion in flow cytometry. In the transfection conditions we have determined (see “Experimental Procedures”), the percentage of transfected cells was about 95% (as evaluated by flow cytometry) with a cell viability at 24 h after transfection between 70% of the total amount of cells (including cells in suspension) and more than 95% of the adherent cells after washes.

Spectrin siRNAs were efficient only after 48 h transfection as evaluated on Western blots; their effects were more pronounced at 72 and 96 h. Among the six siRNAs tested, two were more efficient (siRNAs 1 and 2 from Dharmacon, named Sp1 and Sp2 in Fig. 1, respectively) with a dose-depend-

ent response. At 25 nM concentration, Sp1 siRNA and Sp2 siRNA induced a decrease of at least 50% in the amount of α II-spectrin after 48 h transfection as compared with non-relevant control siRNA (lane NR siRNA), and routinely between 70 and 80% after 72 h transfection.

Spectrin Loss Is Associated with Modifications of Cell Shape and Spreading—We further analyzed the spectrin depletion in transfected cells by immunofluorescence and confocal microscopy. In cells treated with non-relevant siRNA, α II-spectrin antibodies revealed a diffuse distribution of α II-spectrin throughout the cytoplasm, and also an association with the plasma membrane (Fig. 2A). Fibrillar actin labeled with phalloidin was present at the plasma membrane and formed stress fibers (Fig. 2A). These patterns were identical to those observed in non-transfected cells (data not shown). In cells treated with siRNAs targeting α II-spectrin, immunofluorescence staining of α II-spectrin confirmed the decrease of spectrin expression, the residual staining being restricted to the membrane (Fig. 2, B and C). This reduced expression of spectrin was associated with important modifications of the cell shape, the spectrin-depleted cells being much smaller,

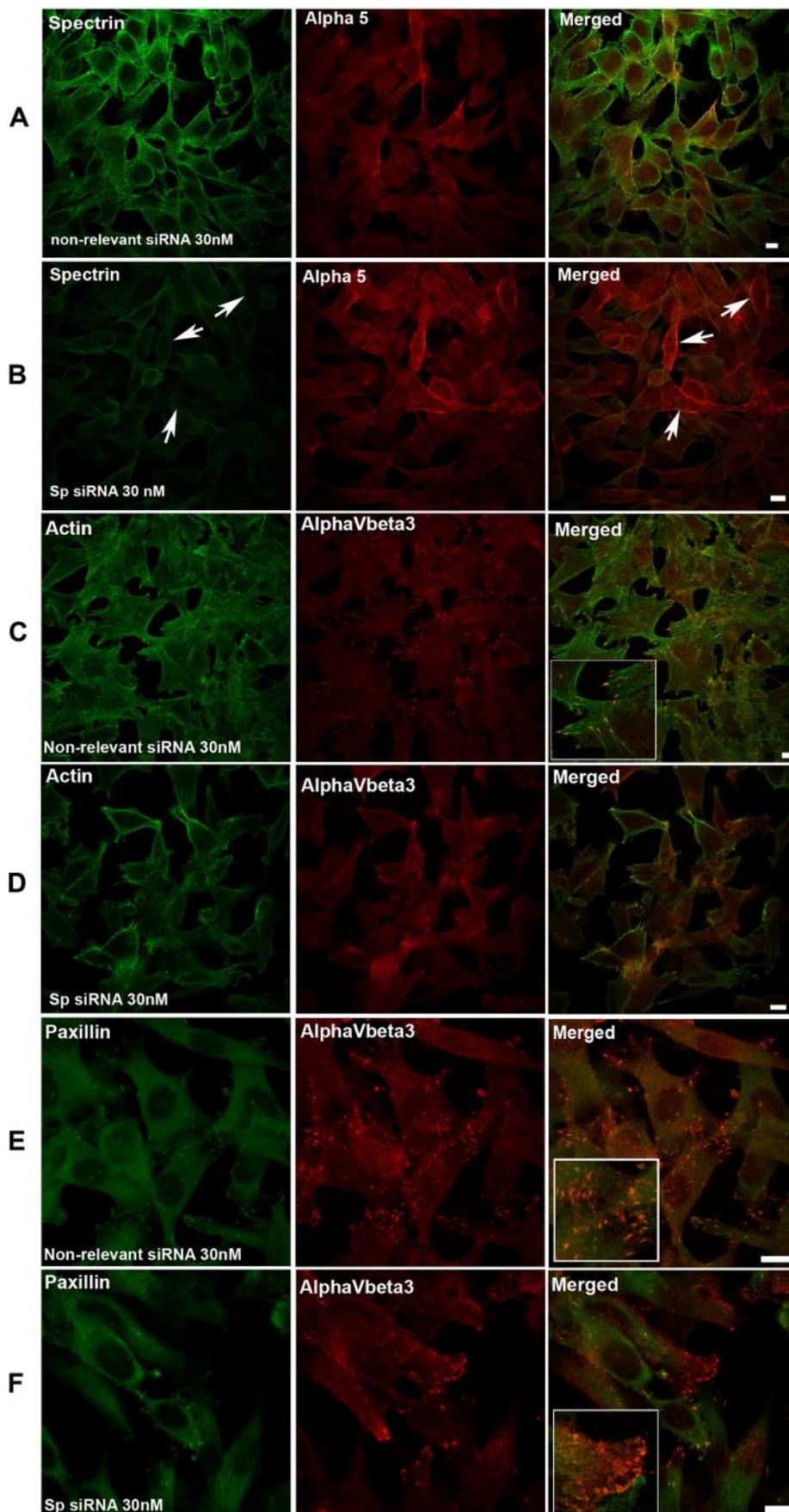
Spectrin Loss Modifies Actin Skeleton

rounded and less spread. Labeling studies with phalloidin showed a disorganization of the actin cytoskeleton, with a reduction of the basal stress fibers network and an increase of the annular cell border (cortical actin). These features were particularly obvious in Fig. 2B where we could compare a non-transfected cell as indicated by its normal spectrin labeling with transfected cells exhibiting a strong decrease in spectrin labeling, reduced size and spreading. Both loss of spreading and actin network disorganization were more pronounced at higher concentrations of spectrin siRNA (Fig. 2C).

During the course of cell culture, we observed a progressive decrease in adherent cells concomitantly to an increase in detached cells in samples treated with spectrin siRNAs, in comparison with control cultures treated either with transfection reagent alone or with non-relevant siRNA. This feature raised two questions: a possible cell death by apoptosis and/or a defect in cell adhesion as suggested by the modifications of cell shape and spreading.

Spectrin Loss Inhibits Cell Proliferation via G₁ Phase Arrest—When compared with samples treated with the transfection reagent alone, the number of total cells (adherent cells and detached cells) was significantly reduced 72 h after transfection in samples treated with siRNA targeting spectrin (Sp1 and Sp2 siRNAs) (50% less) (Fig. 3). This effect was more pronounced after the 96-h culture; the number of cells is 65% less in spectrin siRNA-treated cells. The number of non-relevant siRNA-treated cells was also decreased (25% less after 72 and 96 h transfection), exhibiting a slight toxicity of non-relevant siRNA, but this effect is not statically significant (Fig. 3). The cell numbers observed in samples treated with spectrin siRNAs were significantly decreased compared with samples treated by non-relevant siRNAs ($p < 0.05$ at 96 h after transfection).

This decreased number of total cells could be related either to apo-



ptosis and cell death or to growth arrest. In a first step, we have checked apoptosis and cell death by flow cytometry analysis. Apoptotic cells were identified by the absence of labeling with DiOC₆, a marker of mitochondrial membrane potential, and dead cells were assessed by DNA labeling with propidium iodide. At 72 h after transfection, we observed no important differences between cells transfected with the siRNA of spectrin and those transfected with non-relevant siRNA (Fig. 4A). A moderate apoptosis and cell death appeared only at 96 h after transfection (data not shown). We concluded that apoptosis and cell death are late effects and cannot fully explain the loss of cells observed at 72 h.

We investigated the cell cycle by flow cytometry analysis of cell DNA content after labeling with propidium iodide. At 72 h, the number of cells in the S phase (DNA-synthetic phase) and G₂/M phases (second gap phase/mitosis) is clearly decreased in samples treated with spectrin siRNAs, with a concomitant increase in the G₁ phase (first gap phase) as compared with samples treated with non-relevant siRNA (Fig. 4B). The percentage of cells in G₂/M (estimated from 6 independent experiments) is 8.2 ± 1.2 and $7.4\% \pm 2.1$ in samples treated with Sp1 RNA and Sp2 RNA, respectively, compared with $14.2\% \pm 1.2$ in samples treated with non-relevant siRNA. This effect is more pronounced at 96 h after transfection (data not shown). These data suggest that spectrin depletion is associated with an arrest of cell cycle at G₁ phase.

To better explore the cell cycle regulation, we checked for the phosphorylation state of the retinoblastoma protein (Rb), which regulates cell cycle progression. In resting cells, the activity of Rb is negatively regulated by cyclin-dependent kinases, which phosphorylate Rb in the G₁ phase. Thus the hyperphosphorylated species are primarily found in proliferating cells. As shown by Western blot in Fig. 4C, Rb migrates as multiple bands due to varying degrees of phosphorylation. In cells treated with transfection reagent and non-relevant siRNA, the predominant species consisted of hyperphosphorylated forms (ppRb) (Fig. 4C, lanes 1 and 4, respectively), whereas in cells treated with siRNAs targeting spectrin, the hyperphosphorylated Rb (ppRb) is strongly decreased with a concomitant increase in the underphosphorylated Rb (pRb) (Fig. 4C, lanes 2 and 3, respectively).

This cell cycle arrest was further studied by analyzing the expression of G₁ checkpoint and G₁/S transition proteins, and especially cell cycle inhibitors such as p21, p27, p16, and p15 by Western blot. We failed to detect p16 inhibitor, which is frequently mutated in melanoma cells (17). The p27 inhibitor was also difficult to analyze as its expression is often decreased in melanoma cells. P15 inhibitor expression was not obviously modified (data not shown). In contrast, p21 inhibitor expres-

sion was increased between 1.8 and 2.4 times at 72 h in samples treated with Sp1 and Sp2 siRNAs (at 30 nM) as compared with samples treated with transfection reagent (lane TR in Fig. 4, D and E); p21 expression was slightly increased ($\times 1.2$) in samples treated with non-relevant siRNA (Fig. 4, D and E). These effects were still observed at 96 h (data not shown). To appreciate its role in the cell cycle control in the WM-266 cell line, protein p21 was knocked-down. After 48 h of siRNA treatment at a concentration of 30 nM, p21 was not detected in the cell lysates by Western blotting (Fig. 5A); the p21 loss was associated with a significant increase of cell proliferation, indicating that p21 acts as a negative modulator of proliferation in this cell line (Fig. 5B).

Spectrin Depletion Is Associated with a Defect of Cell Adhesion—The loss of adherent cells transfected with siRNAs targeting α II-spectrin raised the second question of a defect in cell adhesion. As shown in Fig. 2, the cells treated with siRNA targeting spectrin were rounded and not well spread. To address this question, we performed static adhesion assays of melanoma cells at 72 h after transfection as described under "Experimental Procedures." 2 h after replating, the number of adherent cells transfected by non-relevant siRNAs was similar to that of cells treated with transfection reagent (100%) (Fig. 6A). In contrast, the number of adherent cells treated with Sp1 and Sp2 siRNAs was significantly decreased compared with non-relevant siRNA-treated cells: only 40 and 70% of cells treated with Sp1 and Sp2 siRNAs, respectively, were still adherent. In addition to a defective adhesion, the spectrin knock-down cells showed a defect in spreading as shown in Fig. 6, B and C: 4 h after seeding, 24 and 37% of the remaining adherent cells treated with Sp1 and Sp2 siRNAs were spread as compared with 68 and 54% for adherent cells treated with transfection reagent or non-relevant siRNA, respectively.

This defect of cell adhesion prompted us to investigate the abundance of integrins. Integrin $\alpha\beta$ heterodimers bind to a component of the extracellular matrix by their extracellular region and their intracellular region is in relationship with the cytoskeleton, mediating various intracellular signaling pathways. We evaluated from three independent flow cytometric experiments the membrane expression of the main integrins such as $\alpha 3$, αV , and $\beta 1$ chains, which are known to be expressed in melanocytes, melanoma cell lines, and tissues as well, and the integrins that are abnormally expressed in melanoma such as $\alpha 4$, $\alpha 5$, and $\beta 3$ chains (Fig. 7). The expression of $\alpha 4$ integrin remained stable after spectrin depletion and a slight but non-significant increase in $\beta 1$ integrin chain expression was revealed both by flow cytometry and Western blot (Fig. 7). In contrast, membrane expressions of $\alpha 3$ and especially $\alpha 5$ chains were significantly increased (between 2 and 3 times for $\alpha 5$

FIGURE 9. Immunofluorescence studies of integrins in spectrin-depleted cells. Immunofluorescence studies were performed on WM-266 cells treated for 72 h with siRNAs at 30 nM: non-relevant siRNA (panels A, C, and E) and a pool of siRNA 1 and 2 targeted to α II-spectrin (Sp siRNA) (panels B, D, and F). In panels A and B, cells were stained with polyclonal anti- α II-spectrin (pseudo-colored in green) and monoclonal antibody against $\alpha 5$ integrin (pseudo-colored in red). In spectrin siRNA-treated cells (panel B), arrows point out the increased integrin membrane labeling in cells markedly depleted in spectrin. Actin organization (labeled with phalloidin and pseudo-colored in green in panels C and D) was modified in spectrin siRNA-treated cells (panel D) with a loss of stress fibers. $\alpha V\beta 3$ integrin (labeled with monoclonal antibodies directed against the heterodimers and pseudo-colored in red) was distributed as well defined dots (panels C and E). These dots were distributed at the extremities of actin stress fibers (insert in panel C) and were co-localized with paxillin, a marker of focal adhesion (pseudo-colored in green) (inset in panel E). In spectrin knock-down cells, the density of dots labeled with $\alpha V\beta 3$ integrin and paxillin was decreased. These adhesion structures appeared bigger and diffuse (panels D and F). All the images were acquired at the bottom of the cells. Bar = 10 μ m.

Spectrin Loss Modifies Actin Skeleton

chain) and this increase was confirmed by Western blot on total cell lysates. A moderate but significant increase in the $\alpha V\beta 3$ complex was also observed by flow cytometry when Western blots confirmed an increase in the $\beta 3$ integrin signal.

The increased membrane expression of some integrins in spectrin-depleted cells associated with a defect in cell adhesion raise the questions about the involvement of these integrins in adhesion. We investigated the adhesion of cells incubated with blocking antibodies directed against $\alpha 3$, $\alpha 5$, and $\alpha V\beta 3$ integrins prior plating. The data showed in Fig. 8 revealed that these molecules participate in WM-266 cell adhesion as manifested by a significant reduced number of adherent cells.

We further analyzed, by immunofluorescence, the location of integrins whose expression is increased in spectrin-depleted cells (Fig. 9). In comparison with the mainly diffuse cytoplasmic labeling of $\alpha 5$ integrin seen in cells treated with non-relevant siRNA (Fig. 9A), co-labeling of $\alpha 5$ integrin and αII -spectrin in cells treated with spectrin-targeting siRNA showed in many cells, particularly those highly depleted in spectrin, an increase in the integrin membrane labeling (pointed by arrows in Fig. 9B).

Classical focal adhesions, regularly scattered at the cell periphery, are characterized by the co-localization of paxillin and $\alpha V\beta 3$ integrin labeling (see merged control Fig. 9E) and the co-localization of the $\alpha V\beta 3$ integrin with the extremities of actin stress fibers (see merged control in Fig. 9C). Treatment with siRNA targeting αII -spectrin led to an obvious decrease in the density of these adhesion structures, as shown by comparison of the $\alpha V\beta 3$ integrin labeling (pseudo-colored in red) between control cells treated with non-relevant siRNA and cells treated with spectrin-targeting siRNAs. Only few arrow-shaped adhesion structures remained at the extreme cell periphery after specific siRNA treatment (Fig. 9F). F-actin labeling with phalloidin again illustrated the reduction of stress fibers and the reinforcement of cortical actin labeling after specific siRNA treatment (see green labeling in both Fig. 9, C and D).

DISCUSSION

Spectrin Depletion and Cell Adhesion, Shape, and Spreading—The αI - and βI -spectrin chains play an important role in erythrocyte shape and membrane integrity, plasticity, and resistance. Phenotypic analyses of erythroid spectrin deficiencies both in mice and humans have provided strong evidence that a normal skeleton is required to assure these functions (2, 3, 18). Recent studies employing siRNA have revealed that ankyrin-G and βII -spectrin are implicated in epithelial cell polarity where these two proteins collaborate in the formation of lateral membrane (19, 20). Such functions have not been defined for αII -spectrin. For the first time, we show that αII -spectrin is an important actor for non-erythroid cell shape and cell-matrix adhesion: partial αII -spectrin depletion is associated with a loss of cell spreading, a defective adhesion, together with a reduced number of focal points, these being less well organized and not regular.

Focal adhesions, which mediate various intracellular signaling pathways, constitute the site of anchorage of the actin cytoskeleton to the cytoplasmic side of the membrane. These

structures are attached to bundles of actin stress fibers. Our data showing both a loss of stress fibers and modifications of focal adhesions are consistent with the concept that stress fibers and focal adhesions are not only physically linked but also highly interdependent. Thus inhibitors of actin polymerization lead to the destruction of focal adhesions, and inhibition of focal adhesion assembly by blocking integrin-mediated interactions inhibit stress fiber formation (21). Focal adhesions are initially formed via $\alpha\beta$ integrin dimerization and then integrins assemble into multiprotein adhesion complexes that contain a variety of cytoskeletal, adaptor, and signaling proteins.

Bialkowska and co-authors (22) have reported an accumulation of αII -spectrin SH3 domain in integrin clusters. In particular, spectrin is colocalized with $\beta 3$ integrin clusters that initiate attachment of cells and is absent from those that appear at later stage of spreading. As in one hand partial spectrin depletion results in a loss of adhesion and spreading and in another hand, spectrin-based skeleton, termed “accumulator machine” (23, 24) appears to be involved in the expression and right location of membrane proteins, we expected a decreased expression of membrane integrins. Surprisingly, both membrane expression (as evaluated by flow cytometry) and total cell content (as evaluated by Western blots) of integrins are either not modified (such as $\alpha 4$ integrin), or increased (as observed for $\alpha 3$, $\alpha 5$ and $\beta 3$ integrins) although these integrins are implicated in the adhesion process (as demonstrated by blocking antibodies experiments). As spectrin has been reported to be present in the initial integrin $\beta 3$ clusters, it could participate not only in the formation, but also in the dynamics of these clusters; a reduced expression of spectrin could lead to an abnormal and non-efficient accumulation of integrins at the cell surface. In any case our results confirm a critical function of αII -spectrin in the adhesion mechanism.

Spectrin and Actin Skeleton—The modifications of the actin skeleton, mainly the disappearance of stress fibers, observed in spectrin-depleted cells point out the links between both spectrin- and actin-cytoskeletons. Spectrin by its β -subunit bearing an actin binding domain was defined as a cross-linking actin protein. Cells overexpressing the actin binding domain of βII -spectrin lost their typical epithelial morphology and disappeared after 10–14 days in culture (25). Moreover, the SH3 domain of αII -spectrin was demonstrated to bind proteins involved in actin dynamics such as EVL, VASP, and Tes (26, 27). EVL and VASP, two members of the Mena/VASP family, are located in filipodia, lamellipodia, and focal adhesions (28, 29) and also in cell-cell contact as recently reported for the spectrin-VASP complexes (30). Tes, a tumor suppressor, is localized along stress fibers and at focal adhesions and interacts with a variety of cytoskeletal proteins of focal adhesion (such as zyxin, vinculin, and talin as well as Mena, VASP, and EVL) (31, 32). RNA interference knockdown of Tes led to a loss of actin stress fibers (33). Finally, by its SH3 domain, αII -spectrin participate in the activation of the Rho GTPase, Rac (22). The overexpression of the αII -spectrin SH3 domain inhibits Rac activation, actin filament formation, and spreading, this inhibitory effect being abolished by coexpression of constitutively active Rac. So

α II-spectrin could also have a role in the mechanisms regulating actin machinery through several ligands.

Cell Cycle and Spectrin—Previous results showed that inhibition of spectrin function by microinjected spectrin antibodies in blastomeres causes alterations in cell cycle timing (34). In our studies we demonstrated that in spectrin-depleted cells, the cell cycle was stopped at G₁ phase as manifested by a decreased percentage of cells in the S and G₂/M phases and confirmed by the hypophosphorylation of the Rb protein.

Concomitantly to the cell cycle arrest, we observed an up-regulation of p21 expression, a cyclin-dependent kinase inhibitor (CDKI) that is a major player in cell cycle control (35). Protein p21 has been reported to have a dual function in the cell cycle control: it can act as a negative regulator of the cell cycle progression leading to a cell cycle arrest (35); it may act as a positive regulator of cell cycle by stabilizing interactions between CDK4/CDK6 and cyclins. In this context, repression of p21 results in cell cycle arrest (36). In the cell system we used, repression of p21 is accompanied with growth promotion indicating that p21 functions as a CDK inhibitor. So, the increased expression of p21 observed in α II-spectrin-depleted cells is consistent with a cell cycle arrest at the G₁ phase.

It is noteworthy that in a mice model, down-regulation of ELF expression, an isoform of β II-spectrin, confers susceptibility to tumorigenesis: *elf*^{+/-} mutant mice develop frequent tumors associated with a deregulation of cell cycle control at G₁/S transition and defective tumor growth factor- β signaling (37–39). The relative roles of the two spectrin subunits in the cell cycle remain to be elucidated. However, both studies suggest that spectrins must be considered as a new actor in transduction of extracellular signals controlling cell cycle.

Cell Cycle and Cell Adhesion—Besides the activation of cell surface growth factors receptors by soluble mitogens, it is now clear that for a great number of cell types, cell-matrix adhesion is essential for the progression in the cellular cycle (40, 41). Integrin occupation and clustering leads to stimulation of multiple early mitogenic events associated with the transition from G₀ to G₁ phase of the cell cycle.

The fact that in spectrin-deficient cells, cell cycle arrest occurs at the G₁ phase, is consistent with the loss of adhesion observed in these cells. Adhesion through integrins could modulate molecular events required to the progression of cell cycle, as the decrease in CDKI (p21 and p27) (42). Moreover, disruption of the actin cytoskeleton using cytochalasin inhibits S-phase entry with down-regulation of cyclin D1, up-regulation of p27, and inhibition of pRb phosphorylation (43). So, the cell cycle arrest observed in spectrin-depleted cells could be secondarily related to cell adhesion defect.

Conclusion—Taken together, the results presented here provide novel insights into the function of spectrins that can act not only as a structural component, but appear to be involved in signaling pathways. Our data provides a new basis for integrating spectrin in cell organization, and may offer a new mechanism by which changes in spectrin-based cytoskeleton modify the actin reorganization and can influence cell cycle progression. More work is required to gain a clearer picture of the importance of spectrin both in cell adhesion and in actin dynamics and consequently in the regulation of the cell cycle.

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Spectrin Loss Modifies Actin Skeleton

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