Introduction

Many types of mammalian cells require adhesion to the extracellular matrix to proliferate (Assoian and Schwartz, 2001). Integrins are the major family of receptors that mediate cell-matrix adhesion (Hynes, 2002). It is well established that integrins synergize with growth factor receptors to promote the G1–S transition of the cell cycle (Assoian and Schwartz, 2001). Progression through the cell cycle is accompanied by changes in adhesive interactions with the extracellular matrix and the remodeling of the actin and microtubule (MT) cytoskeletons (Glotzer, 2001). During interphase, integrins cluster at matrix contacts called focal adhesions (FAs; Geiger et al., 2001). Actin filaments organize in stress fibers that terminate at FAs, and MTs radiate from the centrosome to the cell cortex (Vandre et al., 1984; Geiger et al., 2001). As mitosis begins, cells loosen attachments; disassemble FAs, stress fibers, and MTs; and adopt a round morphology (Maddox and Burridge, 2003). MTs then realign into the bipolar spindle to direct accurate segregation of genetic material, and actin filaments form the contractile ring to separate daughter cells during cytokinesis (Vandre et al., 1984; Glotzer, 2001). As cell division nears completion, daughter cells respread and FAs, stress fibers, and the radial MT network are reformatted. This dynamic regulation of adhesion during cell division suggests a mechanistic link. A requirement for matrix adhesion for the division of some cell types was reported more than two decades ago (Orly and Sato, 1979; Ben-Ze’ev and Raz, 1981; Winklbauer, 1986). In addition, β1-null chondrocytes exhibit a high incidence of binucleation, suggesting that β1 integrins regulate cytokinesis in this cell type (Aszodi et al., 2003). Here, we report that a mutation in the integrin β1 tail can regulate centrosome function, the assembly of the mitotic spindle, and cytokinesis.

Results and discussion

The conserved membrane-proximal NPXY motif in the β1 tail regulates integrin activation (O’Tooile et al., 1995; Bodeau et al., 2001). To test whether this motif is required for cell proliferation, we generated CHO cell lines stably expressing either a wild-type (WT) β1 tail or a mutant β1 tail with an alanine substitution at tyrosine 783 within the NPIY motif.
In the context of the αIIb-5β3-1 heterodimeric chimeric integrin, these chimeras contain the extracellular and transmembrane domain of the αIIbβ3 fibrinogen (Fg) receptor connected to the tails of the α5β1 fibronectin (Fn) receptor (Fig. 1 A), allowing CHO cell adhesion to Fg (Ylanne et al., 1993). We isolated the function of the recombinant chimeras by adhering cells to Fg in the serum-free growth medium CCM1 that does not support CHO cell proliferation in the absence of a preexisting matrix (unpublished data). WT cells showed robust proliferation on Fg in CCM1, whereas CHO K1 and Y783A cells proliferated poorly (Fig. 1 B). CCM1 similarly promoted proliferation of Y783A and CHO K1 cells on Fn (Fig. 1 B). Furthermore, infection of Y783A cells with an adenovirus that directed the expression of the WT β3-1 chimeric subunit containing the WT β1 tail restored cell proliferation of Y783A cells (unpublished data). Although Y783A cells show slow adhesion kinetics on Fg (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200603069/DC1), most cells adhere and spread...
by 3 h (Fig. S1 B). Thus, the defect in proliferation is not simply due to a lack of adhesion.

Because anchorage-dependent cells require integrin signaling for entry into S phase (Assoian and Schwartz, 2001), we compared cyclin D1 induction and DNA synthesis in WT and Y783A cells. As expected, both were low in serum-starved cells. Surprisingly, cyclin D1 accumulation in Y783A cells at 7 h was equivalent to that in WT cells (Fig. 1 C). Furthermore, WT and Y783A cells incorporated similar levels of BrdU (Fig. 1 D). Thus, the proliferation defect is unlikely to be at the G1–S transition.

The poor proliferation of the Y783A cells was accompanied by accumulation of bi- and multinucleated cells (Fig. 1 E). Analysis of the binucleation kinetics revealed a uniform increase in the nuclei per cell in Y783A cells with no significant change in WT cells (Fig. 1 F). Because the most likely explanation was a defect in cytokinesis, we examined this process by time-lapse microscopy. After rounding at mitosis, WT cells completed cleavage furrow ingression within 5–10 min and cytokinesis within 20–30 min (unpublished data). In contrast, most Y783A cells attempting cytokinesis showed cleavage furrow regression and cytokinesis failure. This phenotype was suppressed when Y783A cells were adhered to Fn (Fig. 1, G and I). Quantification of cytokinesis attempts and successes during the first cell cycle on Fn in CCM1 indicated that ~90% of the WT cells attempted and successfully completed cytokinesis within 16–20 h (Fig. 1, H and I). A significant percentage of Y783A cells attempted cytokinesis, but most failed to divide (Fig. 1, H and I). Many Y783A cells showed a partially constricted cleavage furrow changing in diameter through an extended period of time and finally regressing to produce binucleated cells. In those few cases where cleavage furrow ingression was completed, midbody formation and/or daughter separation was significantly delayed or inhibited (unpublished data). As expected, Y783A cells successfully completed cytokinesis under the same conditions that promoted their proliferation (Fig. 1 H). Together, our data indicate that the Y783A mutation inhibits the successful completion of cytokinesis.

To gain mechanistic insight, we compared the actin and MT cytoskeletons in mitotic WT and Y783A cells that had proliferated on Fg in CCM1 for 15–18 h (a time of peak in cytokinesis attempts; Fig. 1 H). Cells at prometaphase/metaphase were identified by their round morphology and the presence of condensed chromosomes. At this stage, the majority of WT cells (85%) formed functional bipolar spindles, as judged by α- and γ-tubulin distribution and chromosome congression at the equatorial plane (Fig. 2 A). In contrast, most Y783A cells showed random distributions of chromosomes and multipolar spindles or no evidence of spindle assembly (Fig. 2 A). As expected, Y783A cells formed functional bipolar spindles on Fn (Fig. 2 B). At anaphase, WT cells showed normal contractile rings and chromosome segregation (Fig. 2 C); in contrast, most
Y783A cells showed evidence of multiple contractile rings and a lack of chromosome segregation consistent with the presence of aberrant spindles (Fig. 2 C, middle). The few Y783A cells that were able to segregate chromosomes (Fig. 2 C, right) exhibited abnormalities at telophase (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200603069/DC1). Collectively, our data strongly suggest that the Y783A mutation inhibits cytokinesis by preventing the formation of a normal bipolar spindle.

To determine whether the defects were restricted to mitosis, we compared the MT cytoskeletons of Y783A and WT cells at interphase. WT cells had a complex array of numerous, long, distinct polymers originating from the centrosome and radiating to the cell cortex as expected (Fig. 2 D). In contrast, Y783A cells had fewer and more randomly organized MTs, which did not appear to emanate from the centrosome (Fig. 2 D). The Y783A phenotype was not due to decreases in expression of tubulin or surface chimeric integrins (Fig. S1, B and C). However, when we compared MT regrowth after nocodazole washout (Fry et al., 1998), we found that the regrowth of a radial MT array from interphase centrosomes and spindle poles. Results represent the mean ± SD from three independent experiments, each including 200 cells at interphase (D) and 25–50 mitotic cells (F). (G) Time-lapse comparison of cytokinesis in a representative WT (top), one of the common Y783A phenotypes in control IgG-treated cells [middle], and a typical rescue in LIBS6-treated Y783A cells [bottom]. Cells were serum starved and harvested, and antibodies were added before the cells were replated on Fg. Time-lapse images were collected in 3-min intervals (bottom right on each image). The first image was considered time 0. Bar, 20 μm. (H) Quantification of the rescue of cytokinesis by LIBS6. A successful event was scored as described in Fig. 1 H. Results represent the mean ± SD from three independent experiments (≥100 cells/treatment). (I) Rescue of bipolar spindles by LIBS6. Cells were processed as above. At 15–18 h, cells were fixed, stained, and analyzed as described in Fig. 2. Images correspond to representative prometaphase/metaphase and anaphase LIBS6-treated Y783A cells. Bar, 20 μm. (J) Quantification of the rescue of bipolar spindle by LIBS6. Presence of a bipolar spindle was determined as described in Fig. 2 A. Results represent mean ± SD from three independent experiments, each including 25–50 mitotic cells.
CHO cells (O’Toole et al., 1995). Therefore, we tested whether LIBS6, a specific activating antibody for αIIbβ3 (Frelinger et al., 1991), could prevent the mutant phenotypes in Y783A cells. In control experiments, LIBS6 activated the Y783A chimeric integrin (unpublished data) and promoted rapid adhesion and spreading of Y783A cells on Fg (Fig. S1 D). Importantly, LIBS6 rescued the assembly of a radial MT network (Fig. 3, A and B), MT regrowth from centrosomes at interphase (Fig. 3, C and D) and spindle poles at mitosis (Fig. 3, E and F), the assembly of a bipolar spindle (Fig. 3, I and J), and cytokinesis (Fig. 3, G and H; and Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200603069/DC1). In agreement with these results, LIBS6 also prevented binucleation (unpublished data).

To determine whether the effects are specific for the Y783A mutation and the LIBS6 antibody, we tested additional mutations known to regulate integrin conformation (O’Toole et al., 1994, 1995). Coexpression of the αIIb-L deletion (αIIb-LΔ) with the β3-1(Y783A) chimeric subunit resulted in a constitutively active integrin (Fig. S2, D–F) and rescued MT regrowth (Fig. 4), as observed with LIBS6. In addition, coexpression of a β3-1 subunit containing an N780A mutation in the NPIY motif (β3-1[N780A]) with the αIIb-5 subunit prevented soluble Fg binding (Fig. S2, D–F) and inhibited MT regrowth (Fig. 4), mimicking the effects of the Y783A mutation. Thus, the inhibition and rescue of MT regrowth are not specific to the Y783A mutation and the LIBS6 activating antibody.

To demonstrate that the effects of the Y783A mutation were not specific to CHO cells or due to its expression in the context of the αIIb-5β3-1, we generated GD25 cell lines (β1-null; Fässler et al., 1995) expressing full-length human β1 containing either the WT (GD25 h-β1WT) or the Y783A mutant tail (GD25 h-β1Y783A; Fig. 5 A). In contrast, to the GD25 h-β1WT cells, MT regrowth from interphase centrosomes was inhibited in GD25 h-β1Y783A cells adhered to laminin-1 (Lm). Moreover, this phenotype was suppressed by TS2/16 (Masumoto and Hemler, 1993), a specific β1-activating antibody (Fig. 5, B and C). In addition, when these cells were mitotically arrested and replated on Lm, WT but not Y783A cells formed a normal bipolar spindle (Fig. 5, D and E). Thus, the effects of the Y783A mutation are not cell-type or integrin specific.

Our results provide the first evidence that integrins can regulate the assembly of the MT cytoskeleton during interphase and the bipolar spindle during mitosis and indicate that integrin activation is important for both. Previous studies demonstrated that the NPYX motif in β tails regulates activation by binding to talin (Tadokoro et al., 2003). Activating integrin antibodies circumvent the requirement for talin–β tail interactions in integrin activation (O’Toole et al., 1995). The ability of LIBS6 and TS2/16 to rescue the MT regrowth, bipolar spindle formation, and cytokinesis suggests that protein interactions with the NPIY motif, including talin binding, are not required downstream of integrin activation to regulate these processes. These antibodies may promote the association of integrins with other receptors and/or the interaction of the tail with cytoskeletal or signaling proteins to regulate the MT cytoskeleton.
MT dynamics are under tight and complex control throughout the cell cycle. At interphase, the organization of the MT network requires the regulation of MT nucleation, growth, and anchorage at the centrosome and the association of MTs with the cell cortex. The assembly of the bipolar spindle at mitosis also requires the regulation of kinetochore-associated MTs, as well as centrosome duplication and cohesion (Doxsey, 2001; Gadde and Heald, 2004; Kline-Smith and Walczak, 2004; Maiato et al., 2004). The Y783A mutation may inhibit the activity of one or more of the proteins that regulate these events. The goal of future studies will be to identify the aspects and targets of integrin function required for spindle assembly and cytokinesis.

Materials and methods

Cell culture

CHO K1, WT, and Y783A CHO cell lines were cultured in F12 medium ± 10% FBS or CC1M (HyClone). WT and Y783A-GD25 cells were cultured in DME ± 10% FBS or CC1M as indicated. The generation of stable CHO and GD25 cell lines is described in the supplemental text (available at http://www.jcb.org/cgi/content/full/jcb.200603069/DC1). Transient transfection of CHO K1 cells was performed using the Mojo transfection reagent (Invitrogen). Where indicated, cells were serum starved in F12 or DME and replated on the indicated matrices. For binnucleate and cytokinetic analysis, ~5 × 10⁴ cells were replated in CC1M in 24-well dishes on 15 μg/ml Fg/Fn (CHO cells) or 30 μg/ml Lm (GD25 cells) and processed as described in the figure legends.

Cell proliferation assays

Serum-starved cells were replated onto either Fg- or Fn-coated (15 μg/ml) 12-well dishes (3 × 10⁵ cells/well, in triplicate) fixed at the indicated times in 3.7% paraformaldehyde, and stained with crystal violet (0.5% in 20% methanol) for 1 h at room temperature. Incorporated dye was extracted with 1% SDS and quantified by measuring A₅₉₀ in a spectrophotometer.

MT proliferation

Serum-starved cells (~5 × 10⁴) were replated on 15 μg/ml of either Fg or Fn (CHO cells) or on 30 μg/ml Lm (GD25 cells) in CC1M. 3–14 h after plating, cells were treated with 10 μg/ml nocodazole (Calbiochem) for 2 h at 4°C, washed with cold PBS to remove the drug, allowed to nucleate MIs for 5–15 min in warmed CC1M ± Lm (CHO cells) or TS2/16 (GD25 cells), and processed for immunofluorescence microscopy.

Time-lapse microscopy

Serum-starved cells were cultured on 100-mm dishes on 2.5 × 10⁵ cells/ml in CC1M supplemented with 100 μM Hepes, pH 7.4. 3 h after seeding, when most cells were fully spread, dishes were transferred to a microscope equipped with a heated (37°C) chamber, and phase-contrast images were recorded (12 frames/h) during the first cell cycle (16–20 h). Multiple fields (three per sample) were analyzed using a rotary stage and 20× objective. Where indicated, cells were arrested at metaphase by nocodazole treatment, isolated, replated on Fn or Fg in CC1M ± Lm, and imaged as before but at 2 frames/min to analyze cytokinesis in greater detail.

Immunofluorescence microscopy

Cells were permeabilized for 30 s in 80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl₂, and 0.5% Triton X-100; fixed for 10 min in the same buffer containing 5% glutaraldehyde; and incubated for 7 min in 1% sodium borohydride in PBS. To coanalyze MIs and F-actin, cells were fixed in 3.7% paraformaldehyde and 1% sucrose in PBS for 5 min, washed once in PBS, permeabilized in PBS and 0.5% Triton X-100 for 10 min, and processed for immunostaining as described in Materials and methods.Sections were then incubated with primary antibodies, and secondary antibodies coupled to Alexa Fluor 488, 555, or 647 were added.

Online supplemental material

The supplemental text provides information relating to the generation of chimeric integrins and stable cell lines. Fig. S1 shows the adhesion and spreading of WT and Y783A cells on Fg in CC1M, expression levels of α- and β- integrins and chimeric integrins under these conditions, and the promotion of rapid adhesion and spreading of Y783A cells on Fg by Lm. Fig. S2 shows the characteristic telophase phenotypes in these cells under similar conditions (S2A–C), as well as the characterization of the ability of CHO cells transiently expressing WT and mutant chimeric integrins to bind soluble and immobilized Fg (S2D–G). Videos 1 and 2 show the completion and failure of the cytokinesis in WT and Y783A cells, respectively, on Fg in CC1M. Videos 3 and 4 show the rescue of cytokinesis in Y783A cells on Fn or on Fg when treated with Lm, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603069/DC1.

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References


Materials and methods

Generation of IIb-3-1 chimeric integrins

The cDNAs encoding the chimeric αIIb-5 and β3-1 subunits in pCDM8 (provided by Dr. M. H. Ginsberg, University of California, San Diego, La Jolla, CA) were further subcloned into pcDNA3.1(+)/neo and zeo (Invitrogen) to generate pcDNAαIIb-5, pcDNAβ3-1(+)WT, pcDNAβ3-1(Y783A), and pcDNA3.1(N780A) as follows. To construct pcDNAαIIb-5, a XbaI fragment containing the chimeric αIIb-5 subunit was purified from pCDM8 and further subcloned into the XbaI site of pcDNA3.1(+)/neo (Invitrogen). Vectors pcDNAβ3-1(+)WT, pcDNAβ3-1(Y783A), and pcDNAβ3-1(N780A) encoding WT and mutant β3-1 subunits were generated in steps. Initially, a HindIII fragment containing a chimeric β3-1 subunit (in which the C-terminal 33 residues of the β1 tail replace those of the β3 tail) from pCDM8 was subcloned into the HindIII site of pcDNA3.1(+)/neo, generating pcDNAβ3-1. Vectors encoding the WT and Y783A mutant β3-1 subunits were generated by ligating three fragments: (1) pcDNAβ3-1 lacking sequences between the BamHI site located within the β3 extracellular domain and the XhoI site downstream of the β1 tail sequence; (2) a 5’-BamHI–HindIII–3’ fragment containing the β3 sequence between the BamHI site and the entire transmembrane domain, generated by PCR with primers 5’-tgctgtgatcctgctgt-3’ (containing the 5’-BamHI site) and 5’-gtgagaagctttcagatgagcagggcggc-3’ (introducing a novel 3’-HindIII site immediately downstream of the transmembrane domain sequence by silent mutation); and (3) a HindIII–XhoI fragment encoding either the entire WT or a Y783A mutant β1 tail, isolated upon double digestion of plasmids encoding tac–β1 and tac–β1(Y783A) chimeric proteins, respectively (Bodeau et al., 2001). The mutant N780A β1 tail was generated by hybridizing eight overlapping oligonucleotides designed to include the N780A mutation, a novel AgeI site (by silent mutations at residues Thr-777 and Gly-788), as well as HindIII and XhoI sticky ends. The fragment was phosphorylated at its unligated 5’ ends using T4 polynucleotide kinase and inserted into the HindIII and XhoI sites of the pcDNA3.1(WT), from which the WT β1 tail had been removed.

Generation of WT- and Y783A-CHO and GD25 cell lines

CHO-WT and Y783A cell lines were generated by cotransfection of parental CHO K1 cells with pcDNAαIIb-5 and either pcDNAβ3-1(WT) or pcDNAβ3-1(Y783A) followed by the selection of stable transfecants by antibiotic resistance in F12 medium containing 10% FBS. Cell populations expressing similar surface levels of the heterodimeric chimeric integrins were purified by fluorescence-activating cell sorting using the FITC-conjugated CD41 antibody (Immunotech). GD25-WT and -Y783A cell lines were generated by stable transfection of the GD25 cell line (provided by R. Fassler, Max Planck Institute for Biochemistry, Martinsried, Germany) with either the full-length human WT/Y783A β1 integrin and pcDNAIIb-5, a XbaI fragment containing a chimeric IIb-5 subunit was purified from pCDM8 and further subcloned into the XbaI site of pcDNA3.1(+)/neo and zeo (Invitrogen). The full-length cDNA for the human β1 integrin was provided by Y. Takada (University of California, Davis, Davis, CA). Similar expression of the human WT and Y783A mutant β1 integrin was confirmed by flow cytometry with the anti-human β1 integrin antibody (mAb K20; Biomedica).

WT β3-1 adenovirus

The recombinant adenoviral vector pAdβ3-1WT was generated using the AdEasyTM system (He et al., 1998). The WT β3-1 cDNA was first subcloned into the HindIII site of pAdTrack-CMV, between the CMV promoter and an internal ribosome entry site (IRES) upstream the EGFP sequence. The expression cassette was transferred into the adenoviral genome in vector pAdEasy-1 by homologous recombination in BJ5183 cells. Recombinants were identified by restriction enzyme analysis. Transfection-quality pAdβ3-1 was linearized with Pac I and transfected into QBI-293A cells using Escort IV transfection reagent (Sigma-Aldrich). The amplified recombinant adenovirus was purified on CsCl gradients.

References

