Phosphorylation of the Integrin α4 Cytoplasmic Domain Regulates Paxillin Binding*

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α4 integrins are essential for embryogenesis, hematopoiesis, inflammation, and immune response possibly because α4 integrins have distinct signaling properties from other integrins. Specifically, the α4 cytoplasmic domain binds tightly to paxillin, a signaling adaptor protein, leading to increased cell migration and an altered cytoskeletal organization that results in reduced cell spreading. The α4 tail contains potential phosphorylation sites clustered in its core paxillin binding region. We now report that the α4 tail is phosphorylated in vitro and in vivo. Furthermore, Ser988 is a major phosphorylation site. Using antibodies specific for Ser988-phosphorylated α4, we found the stoichiometry of α4 phosphorylation varied in different cells. However, >60% of α4 was phosphorylated in Jurkat T cells. Phosphorylation at Ser988 blocked paxillin binding to the α4 tail. A phosphorylation-mimicking mutant of α4 (α4S988D) blocked paxillin binding and reversed the inhibitory effect of α4 on cell spreading. Consequently, α4 phosphorylation is a biochemical mechanism to modulate paxillin binding to α4 integrins with consequent regulation of α4 integrin-dependent cellular functions.

Integrin adhesion receptors are heterodimers of α and β subunits comprised of a large extracellular domain responsible for ligand binding, a single transmembrane domain, and a cytoplasmic domain that in most cases consists of 20–70 amino acid residues (1, 2). Integrins mediate cell adhesion and participate in cell migration and cytoskeletal re-organization (1, 3). The α4 integrins are expressed on leukocytes and their precursors, neural crest cells, and in developing skeletal muscle (4, 5). They are essential for embryogenesis, hematopoiesis, and immune responses (4–7). The α4 integrin subunit regulates cell migration, cytoskeletal organization, and gene expression in a distinct manner from other integrin α subunits (8). α4 integrins promote cell migration and antagonize cell spreading and contractility. These biological activities depend on the α4 cytoplasmic domain (8). Indeed, this α4 tail markedly stimulates cell migration and opposes cell spreading and focal adhesion formation when joined to other integrin α subunits (8).

We previously used model proteins to mimic clustered integrin cytoplasmic domains (9). Integrin tails are tethered at their N terminus to membranes spanning presumptive α-helices. More importantly, they have vertical constraints, since they are initially parallel to each other and are in a specific vertical stagger as they exit the membrane. In the model proteins, clustering was mimicked by use of covalent homodimers of these domains. Helical coiled coil architecture provided the desired parallel topology and vertical stagger of the tails. Using these model proteins, we found that paxillin, a cytoplasmic signaling adaptor protein, bound tightly to the α4 cytoplasmic domain (10). Mutations in a core nanopeptide paxillin binding sequence disrupt paxillin binding and block the ability of the α4 tail to promote migration, oppose cell spreading, and alter cytoskeletal organization (10, 11). Furthermore, α4β1-dependent adhesion to one of its ligands, vascular cell adhesion molecule-1 (VCAM-1), led to spreading of mouse embryonic fibroblasts derived from paxillin-null but not from wild-type mice (10). Consequently, the α4-paxillin interaction plays an important role in the unusual signaling properties of α4 integrins.

Cell migration requires rapid temporal and positional modulation of integrin-dependent cellular functions (12–14). Therefore, the importance of the α4-paxillin interaction in cell migration suggested that it might be subject to regulation by cellular signaling events. Phosphorylation-dephosphorylation reactions are among the most widely used signaling mechanisms. Furthermore, the core paxillin binding sequence of the α4 tail contains several potential phosphorylation sites (see Fig. 1A), leading us to assess its potential phosphorylation. In the present study, we identified phosphorylation of the α4 tail in vitro and in vivo, mapped a major phosphorylation site to Ser988, and prepared antibodies specific for phosphorylated α4. Stoichiometries of phosphorylation varied widely in different cell types. However, in Jurkat T cells >60% of surface α4 was phosphorylated as assessed with the phospho-specific anti-α4 antibody. Phosphorylation at α4 Ser988 inhibited paxillin binding to the α4 tail and its physical association with the α4β1 integrin. A phosphorylation-mimicking mutant of α4 (α4S988D) blocked paxillin binding and reversed the inhibitory effect of α4 on cell spreading. Consequently, phosphorylation of the α4 tail at Ser988 is a novel biochemical mechanism to modify α4 integrin-dependent cellular events.

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EXPERIMENTAL PROCEDURES

Materials and DNA Constructs—ATP and protein kinase A were purchased from Sigma. Modified trypsin (TPCK-trypsin) was from Promega (Madison, WI). Sulfo-NHS-biotin was from Pierce. Vectastain ABC kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). ECL Western blotting detection kit was from Amersham Pharma-
cia Biotech. [γ-32P]ATP and [32P]-inorganic phosphate were from PerkinElmer Life Sciences. The mammalian expression vector for human β1 integrin (pHSkβ1a) was a generous gift from Dr. Y. Shimizu (University of Minnesota, Minneapolis, MN). Bacterial expression vector for HA-tagged glutathione S-transferase (GST)-paxillin protein (1.7×pGEK) was kindly provided by Drs. R. Salgia and James Griffith (Dana-Farber Cancer Center, Boston, MA). HA-tagged GST-paxillin was purified as described before (11, 15). Rabbit polyclonal antibodies specific for the cytoplasmic tail of integrin α6 (Rb3083) and for paxillin (Rb4356) were raised against the cytoplasmic tail of α6 (KAGFFKRQKYLQICENRDSWSYINSKND) conjugated to keyhole limpet hemocyanin and GST fusion of paxillin expressed from 1.7×pGEK (16), respectively, and further purified by affinity chromatography using protein G-Sepharose column (Amersham Pharmacia Biotech). The following antibodies were obtained commercially: monoclonal antibody against human α6 (HP2/1, Immunotech, Marseille, France), against paxillin (clone 124, Transduction Laboratories, Lexington, KY), against HA tag (12CA5, ATCC), and against GST (B-14, Amersham Pharmacia Biotech). Human lymphocytes were purified from peripheral blood by centrifugation at 16,000 × g for 15 min at 4°C. Clarified lysates were preclared with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. α4 was then immunoprecipitated by the incubation of lysate from 2 × 10^7 cells with 1 μg of anti-α4, monoclonal antibody (HP2/1, Immunotech) for

leupeptin, 0.7 μg/ml pepstatin, 0.5 mM Fabsicol) and phosphatase inhibitors (20 mM glycophosphate, 50 μM sodium vanadate, 1 mM NaF, and 10 μM p-nitrophenyl phosphate) for 3 h and washed several times in HBBS with protease inhibitors and phosphatase inhibitors. Twenty-minute kinase reactions at 30°C were initiated by the addition of 3 μl of kinase buffer containing [γ-32P]ATP (6000 Ci/mmol) and 40 μM ATP. The bead-bound recombinant tail was then washed several times with ice-cold HBBS, boiled in SDS-PAGE sample buffer, and resolved by 4–20% SDS-PAGE under reducing conditions. 32P-labeled recombinant tail mimic proteins were visualized after autoradiography.

Metabolic Cell Labeling—After washing with phosphate-free medium, cells were incubated for 4 h at 37°C in phosphate-free medium containing 10% dialyzed fetal bovine serum and 0.3 mM/mL [32P]orthophosphate (PerkinElmer Life Sciences). Cells were washed several times with ice-cold phosphate-free medium. Cell lysates and α4 immunoprecipitates were prepared as described below and analyzed by SDS-PAGE followed by autoradiography and Western blotting.

Immunoprecipitation and Western Blotting—Cell lysates were routinely prepared with Nonidet P-40 lysis buffer (20 mM HEPES, pH 7.5, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl2, 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mM Fabsicol, 20 mM glycophosphate, 50 μM sodium vanadate, 1 mM NaF, 10 μM p-nitrophenyl phosphate). For co-immunoprecipitation experiments, SL lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, Triton X-100, 0.05% Tween 20, 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mM Fabsicol, 20 mM glycophosphate, 50 μM sodium vanadate, 1 mM NaF, 10 μM p-nitrophenyl phosphate) was used. Lysates were held in melting ice for 30 min, and insoluble material was pelleted by centrifugation at 16,000 × g for 15 min at 4°C. Clarified lysates were preclared with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. α4 was then immunoprecipitated by the incubation of lysate from 2 × 10^7 cells with 1 μg of anti-α4, monoclonal antibody (HP2/1, Immunotech) for

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unlabeled α4 integrin (pHsk

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1 integrin (pHsk

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1 tail mimic proteins and isolated by reverse phase performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.

The presence of the desired mutation was verified by sequencing, and the HindIII-BamHI fragment was then subcloned into the modified PET15b (9) expression vector as a HindIII-BamHI fragment. For construction of mutant α6s in mammalian expression vector, pCDNA3.1(+–) vector (Invitrogen, Carlsbad, CA) encoding wild-type α4 was mutagenized using the QuikChange mutagenesis kit. Point mutations were confirmed by sequencing.

Paritition of Recombinant Integrin Cytoplasmic Domains—Production of recombinant model proteins containing the α6 tail has been described (9). Briefly, each recombinant protein was expressed in BL21(DE3)pLysS cells (Novagen, Madison, WI), isolated by Nf'2-charged resins, and further purified to >90% homogeneity using a reverse-phase C18 HPLC column (Vydac, Hesperia, CA). α6/β1A heterodimer tail mimic protein was prepared by oxidation of equimolar mixture of α6 and β1A tail mimic proteins and isolated by reverse phase C18 HPLC. The mass of all recombinant α6 tail proteins were determined by electrospray ionization mass spectroscopy (APPI, FE SCIECIO, Tokyo, Japan) and varied by less than 0.1% from that predicted by the desired sequence.

Cell Lines and Transfections—Human T cell lines (Jurkat, CEM, and HuT78) and monocytic cell lines (U-937 and THP-1) were obtained from the American Type Culture Collection (ATCC). K562 cells expressing α6,β6 were generously provided by Dr. M. Hemler (Dana Farber Cancer Center, Boston, MA). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units of penicillin/ml, 50 μg of streptomyein sulfate/ml, 2 mM l-glutamine, and 1% nonessential amino acids. Human lymphocytes were purified from peripheral blood from normal donors by centrifugation through a Ficoll-Paque gradient (Amersham Pharmacia Biotech) as previously described (15). Chinese hamster ovary cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% nonessential amino acid (Sigma), penicillin, and streptomycin and transiently transfected with the wild-type or mutant human α6 constructs described above along with human β1 construct using LipofectAMINE reagent (Invitrogen, Carlsbad, CA).

In vitro Phosphorylation of Integrin α6—1 μg of recombinant tail model proteins bound to Nf'2-agarose were incubated with cell lysate from Jurkat cells (50 μg of total protein) in HBB (20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM MgCl2, 0.05% Triton X-100) with protease inhibitor mixture (2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml ATP. The bead-bound recombinant tail was then washed several times with ice-cold HBBS, boiled in SDS-PAGE sample buffer, and resolved by 4–20% SDS-PAGE under reducing conditions. 32P-labeled recombinant tail mimic proteins were visualized after autoradiography.

FIG. 1. Integrin α4 is phosphorylated on its cytoplasmic tail in vitro and in vivo. A, schematic representation of the α4 tail (residue 988–999). All the possible putative phosphorylation sites are indicated as bold and underlined characters. Previously mapped paxillin binding motif (11) is boxed. B, recombinant tail mimic proteins of α6 or α6β6 along with α6β6 immobilized on Nf’2-agarose beads were phosphorylated in vitro by incubate from Jurkat cells as described under “Experimental Procedures” and subjected to SDS-PAGE. Phosphorylated proteins were detected by autoradiography. C, phosphorylation of α6 in various cells. Immunoprecipitated (IP) α6 proteins from the 32P-labeled cells were subjected to SDS-PAGE and visualized by autoradiography. α6 was identified by Western blotting using anti-α6 antibody (Rb3083). Note that two α6 bands appeared in the α6 blot; however, the intensity of these two bands did not correlate with the extent of α6 phosphorylation. The lower band is probably an α6 precursor, since it is not observed in immunoprecipitates formed with anti-α6 antibodies from surface-labeled cells (data not shown).

1 The abbreviations used are: TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, TBS with Tween; PKA, protein kinase A; HA, hemagglutinin; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.
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**Fig. 2. Integrin α4 is phosphorylated on Ser988.** A, phosphorylated α4 proteins, immunoprecipitated from 32P-labeled Jurkat cells (left panel) or recombinant α4 tail mimic proteins phosphorylated in vitro in the presence of (γ-32P)ATP were separated by SDS-PAGE and transferred to the membrane. After exposure to film, the band corresponding to α4 was excised and hydrolyzed in 6 N hydrochloric acid for 1 h at 110 °C. Phosphoamino acids were separated by two-dimensional electrophoresis as described above. Experimental Procedures. B, recombinant integrin α4, in vitro phosphorylated by PKA (left panel) or immunoprecipitated α4 from 32P-labeled Jurkat cells (right panel), were subjected to phosphopeptide mapping. Phosphorylated proteins were separated by SDSPAGE, transferred to a nitrocellulose membrane, and visualized by autoradiography. α4 bands were excised and digested with trypsin, followed by two-dimensional mapping using electrophoresis, pH 1.9, in the first dimension and ascending chromatography in the second dimension as described under “Experimental Procedures.” D, recombinant tail mimic proteins of wild type and α4/S988A along with αβm immobilized on Ni²⁺-agarose beads were phosphorylated by either recombinant PKA or Jurkat cell lysate in vitro and subjected to SDS-PAGE. Phosphorylated α4 was visualized by autoradiography as described above.

2 h at 4 °C followed by incubation with goat anti-mouse IgG-Sepharose for at least 2 h at 4 °C. Immunoprecipitates were washed several times in the same lysate buffer, boiled in 1× SDS-PAGE sample buffer, then separated by 4–20% SDS-PAGE (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to either nitrocellulose membrane (Bio-Rad) (100 V, 1 h 30 min) or polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were washed twice in Tris-buffered saline with 0.1% Tween 20 (TBST), blocked with 5% nonfat dry milk in TBST, and incubated with antibodies for 2 h. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies and visualized with ECL chemiluminescence reagents (Amersham Pharmacia Biotech). For re-probing with other antibodies, blots were stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 10% v/v 2-mercaptoethanol, 2% SDS) at 65 °C for 30 min. After washing several times in TBST, the membranes were blocked with 5% nonfat dry milk containing TBST and reprobed as described above. To label surface molecules, cells were washed three times with phosphate-buffered saline and then incubated with 0.5 mg/ml of sulfo-NHS-biotin (Pierce) for 30 min at room temperature. Unreacted biotin was quenched and washed from the cells with PBS (0.1 M Tris-HCl, pH 7.4, 150 mM NaCl). Biotinylated proteins were separated by 3D-PAGE gel, transferred to nitrocellulose membrane, and detected using Vectastain ABC kit and ECL chemiluminescence.

**Fig. 3. Phosphorylation of α4 cytoplasmic tail on Ser988 inhibits paxillin binding to α4.** A, recombinant α4 tail mimic protein bound to Ni²⁺-agarose was phosphorylated by PKA in vitro as described above. Some of the phosphorylated α4 protein was then subjected to in vitro phoshatase reaction using alkaline phosphatase (AP) for 30 min at 30 °C in phosphatase buffer (50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂). Phosphorylated or dephosphorylated α4 tail model protein was incubated with GST-paxillin in PN lysis buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 150 mM sucrose, 50 mM NaF, 40 mM sodium pyrophosphate, 1 mM sodium vanadate, 1% Triton X-100, and protease inhibitor mixture). After a 30-min incubation at room temperature, unbound protein was washed, and pellets were subjected to SDS-PAGE. GST-paxillin was detected by Western blotting using anti-HA antibody (GST-paxillin also has an HA tag). B, 32P-labeled Jurkat cells were surface-biotinylated and lysed with SL lysis buffer (described under “Experimental Procedure”) in the presence or absence of phosphatase inhibitors (20 mM glycerophosphate, 1 mM NaF, 10 mM p-nitrophenol phosphate, 50 μM sodium vanadate). Then paxillin was immunoprecipitated (IP), and commonprecipitated α4 was visualized by blotting for biotin after separation in SDS-PAGE gel (upper panel). Immunoprecipitated paxillin was detected by anti-paxillin antibody. C, α4 was immunoprecipitated from the same Jurkat cell lysate used in panel B and resolved in an SDS-PAGE gel. Phosphorylated α4 was detected by autoradiography and its identity was verified by Western blotting for α4 (Rb038) (lower panel).
Intensifying screens.

Extracellular domain of H9251 TPCK-trypsin (in 0.1 mM HCl) in 200 μL SDS-PAGE, and phosphorylation of H9251 was then exposed to film to localize and electro-transferred to nitrocellulose membrane. The membrane was then cut out and incubated with 0.5% polyvinylpyrrolidine 360 KDa. Phospho-peptide mapping was performed as described by Boyle et al. (19). Briefly, labeled α4 was immunoprecipitated from 32P-labeled cells and separated in SDS-PAGE as described above. Membrane fragments were washed five times with water and finally subjected to SDS-PAGE and then visualized by chemiluminescence after staining with Vectastain ABC (Pierce) along with a phosphoamino acid standards mixture on glass-backed cellulose plates and then phosphorylated by PKA in vitro for the indicated incubation times in the presence of [γ-32P]ATP. After washing, bead-bound proteins were resolved in SDS-PAGE, and phosphorylation of α4 was detected either by Western blotting using phospho-specific rabbit polyclonal anti-α4 antibody (α4*) or by autoradiography. In a similar experiment (left panel), a GST fusion protein, which contains a recognition site for PKA, was phosphorylated, and its phosphorylation was visualized by autoradiography and Western blotting with phospho-specific rabbit anti-α4 antibody. B, specificity of Ser4 phosphorylation-specific antibody by Western blotting of immunoprecipitated (IP) α4. α4 was immunoprecipitated from 32P-labeled Jurkat cells. After washing pellets, immunoprecipitates were resuspended in phosphatase buffer and incubated for the indicated times with alkaline phosphatase (AP) at 30 °C. α4 immunoprecipitates were subjected to SDS-PAGE, and phosphorylation of α4 was visualized by Western blotting with phospho-specific anti-α4 antibody (α4*) or by autoradiography. The presence of α4 in the immunoprecipitates was assessed by Western blotting with an anti-α4 antibody (Rb038) directed against the α4 tail. C, estimation of stoichiometry of α4 phosphorylation. Lysates of surface-biotinylated Jurkat cells were prepared in the presence or absence of phosphatase inhibitors. The lysates were precleared by immunoprecipitation with either phospho-specific anti-α4 antibody (PSα4) or irrelevant rabbit IgG, and the remaining α4 was immunoprecipitated by an antibody reactive with the extracellular domain of α4 (HP2/1). Immunoprecipitates were resolved in SDS-PAGE, and biotinylated α4 immunoprecipitates were subjected to SDS-PAGE, and phosphorylation of α4 was visualized by Western blotting with phospho-specific anti-α4 antibody (α4*) or by autoradiography. The presence of α4 in the immunoprecipitates was assessed by Western blotting with an anti-α4 antibody (Rb038) directed against the α4 tail. C, estimation of stoichiometry of α4 phosphorylation. Lysates of surface-biotinylated Jurkat cells were prepared in the presence or absence of phosphatase inhibitors. The lysates were precleared by immunoprecipitation with either phospho-specific anti-α4 antibody (PSα4) or irrelevant rabbit IgG, and the remaining α4 was immunoprecipitated by an antibody reactive with the extracellular domain of α4 (HP2/1). Immunoprecipitates were resolved in SDS-PAGE, and biotinylated α4 was detected by transfer to nitrocellulose membranes and staining with Vectastain ABC (upper panel). Depicted is the result of one of two experiments with similar results. Densitometry was used for quantitative comparisons (lower panel). α4* indicates phosphorylated α4.

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Rated by two-dimensional thin layer electrophoresis (HTLE 7000, CBS Scientific) along with a phosphoamino acid standards mixture on glass-backed cellulose thin layer chromatography plates (EM Science) using 0.05 M formic acid and 1.36 M glacial acetic acid, pH 1.9, in the first dimension and 0.87 M glacial acetic acid, 0.5% (v/v) pyridine, and 0.5 mM EDTA, pH 3.5, in the second dimension. After visualization of the markers by ninhydrin spraying, the plate was exposed to film (19). Phospho-peptide mapping was performed as described by Boyle et al. (19) and Luo et al. (20). Briefly, labeled α4 was immunoprecipitated from 32P-labeled cells and separated in SDS-PAGE as described above and electro-transfered to nitrocellulose membrane. The membrane was then exposed to film to localize α4. The band corresponding to α4 was then cut out and incubated with 0.5% polyvinylpyrrolidone 360 KDa (PVP360, Sigma) dissolved in 0.1 M acetic acid for 30 min at 37 °C. Membrane fragments were washed five times with water and finally with freshly made 50 mM NH4HCO3 pH 8.0, and treated with 10 μg of TPCK-trypsin (in 0.1 mM HCl) in 200 μL of 50 mM NH4HCO3 overnight at 37 °C. For complete digestion, an additional 10 μg of TPCK-trypsin was added, and the tube was incubated for another 3 h. After the incubation, the remaining NH4HCO3 was removed by repeated lyophilization in a SpeedVac, and the peptides were oxidized by incubation in performic acid. Phospho-peptides dissolved in a small volume of distilled water were spotted on a glass-backed cellulose plate and then separated in the first dimension by electrophoresis, pH 1.9, and separated in the second dimension by ascending chromatography in phos- pho-chromatography buffer (21). The phospho-peptides were visualized by exposing plates to Kodak XAR MS film for 3 days at −80 °C with intensifying screens.

Generation of Phospho-specific Anti-α4 Antibody—The peptide RDS999WSYINSK was synthesized with or without a phosphorylation at Ser9. Both peptides were purified by reversed-phase HPLC, and their identities were confirmed by mass spectrometry. The synthetic peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde as the coupling reagent. Rabbits were immunized by intracutaneous injection of the conjugate and were bled at bi-weekly intervals. Antibodies were rendered phospho-specific by absorption with the α4 tail model protein immobilized on NHS+ chelate resin. Specificities of the final antibodies were verified by immunoblotting.

Measuring the Stoichiometry of Ser Phosphorylation—Jurkat cells were surface-biotinylated by sulfo-NHS-biotin as described above, and phosphorylated α4 was quantitatively precipitated from the Jurkat cell lysate in a preclearing step either with PSα4 antibody or with rabbit IgG. Afterward, the remaining α4 in the cell lysate was immunoprecipitated with HP2/1. Immunoprecipitated α4 was separated in a SDS-PAGE gel (4–20%) and transferred to nitrocellulose membranes and visualized by chemiluminescence after staining with Vectastain ABC. Densitometry (AlphaImager 2000, Alpha Innotech Corp.) was used for quantitative comparisons.

Cell Spreading Assay—Cell spreading was performed as described (10, 11). Briefly, Chinese hamster ovary cells were transiently transfected with wild type or S988D mutant human integrin α4 in combination with human β1. Equal expression of mutant and wild-type α4 integrins was observed by fluorescence-activated cell sorter analysis. Cells resuspended in Dulbecco’s modified Eagle’s medium with 1% bovine serum albumin were plated on coverslips coated with 5 μg/ml recombinant CS-1, an α4 tail model protein immobilized on NHS+ chelate resin. Cells exhibited flattening and the presence of lamellipodia were scored as spreading cells. Digital images were acquired with an Olympus IX70 microscope equipped with CoolSnap digital color camera.
The data represent the mean cells were enumerated as described under "densitometry, and background binding to the was detected by Western blotting. Bound paxillin was quantified by bound proteins were fractionated by SDS-PAGE, and bound paxillin/wt or mutant Immobilized wild-type (wt) or mutant was incubated with GST-paxillin in PN lysis buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 150 mM sucrose, 1% Triton X-100, and protease inhibitor mixture) for 30 min at room temperature. The N15F bead-bound proteins were fractionated by SDS-PAGE, and bound paxillin was detected by Western blotting. Bound paxillin was quantified by densitometry, and background binding to the substrate was subtracted in each experiment. The data represent the mean ± S.D. of three separate experiments. B, effect of S988D mutation on cell spreading. Wild type and mutant Chinese hamster ovary transfected cells were allowed to spread on the CS-1 fragment of fibronectin for 2 h. Images of typical fields were acquired at a magnification of 400× (upper panels). Spread cells were enumerated as described under “Experimental Procedures.” The data represent the mean ± S.D. of triplicate determinations (lower panel).

RESULTS AND DISCUSSION

Phosphorylation of the Integrin α4 Subunit Cytoplasmic Domain—The α4 integrin cytoplasmic domain contains multiple potential phosphorylation sites. To investigate possible α4 phosphorylation, we first assessed the capacity of cell extracts to phosphorylate the α4 tail in vitro. A Jurkat T cell extract phosphorylated the α4 cytoplasmic domain buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 150 mM sucrose, 1% Triton X-100, and protease inhibitor mixture) for 30 min at room temperature. The N15F bead-bound proteins were fractionated by SDS-PAGE, and bound paxillin was detected by Western blotting. Bound paxillin was quantified by densitometry, and background binding to the substrate was subtracted in each experiment. The data represent the mean ± S.D. of three separate experiments. B, effect of S988D mutation on cell spreading. Wild type and mutant Chinese hamster ovary transfected cells were allowed to spread on the CS-1 fragment of fibronectin for 2 h. Images of typical fields were acquired at a magnification of 400× (upper panels). Spread cells were enumerated as described under “Experimental Procedures.” The data represent the mean ± S.D. of triplicate determinations (lower panel).

To assess in vitro phosphorylation of α4, we metabolically labeled Jurkat T cells with [32P]orthophosphate. Immunoprecipitated α4 was biosynthetically phosphorylated in these cells (Fig. 1C). We examined several T cell lines and observed constitutive α4 phosphorylation in Jurkat and CEM (Fig. 1C). α4 was also phosphorylated in all of the monocytic cells that we examined (U937 and THP-1) and in peripheral blood T cells as well (data not shown). Furthermore, treatment of Jurkat cells with agonists such as anti-CD3+ anti-CD28 or phorbol myristate acetate did not increase α4 phosphorylation (data not shown). However, several cell lines exhibited little or no α4 phosphorylation. These included HuT78, rat basophilic leukemia, and α4-transfected K562 cells. Nevertheless, the α4 cytoplasmic domain was present in α4 immunoprecipitates formed from these cells (Fig. 1C). Thus, the α4 tail is constitutively phosphorylated in vivo in some cells but not in others.

Serine Phosphorylation of α4 Integrim—To map the phosphorylation site(s) of α4, we first carried out phosphoamino acid analysis of metabolically labeled α4 immunoprecipitated from Jurkat T cells (Fig. 2A, left panel) and of recombinant α4 tail labeled by an in vitro kinase reaction (Fig. 2A, right panel). In both preparations, only phosphoserine was detected (Fig. 2A). Thus, the α4 tail is phosphorylated on serine residues in vitro and in vivo.

The α4 tail contains 5 serine and 2 tyrosine potential phosphorylation sites (Fig. 1A). A search of the data base of consensus sequence motifs for the potential phosphorylation sites in the α4 tail using PBASE (www.cbs.dtu.dk/services/phase-predict.pl) identified an exact recognition site for protein kinase A (PKA) (XRRXSX, where X indicates any amino acid, X indicates a hydrophobic residue, and underlining indicates the site of phosphorylation) around Ser988 (RRDSWS) (22). To learn whether α4 was a PKA substrate, we assessed the capacity of PKA to phosphorylate the α4 tail in vitro. Both α4 and the αβ1, heterodimer cytoplasmic domains were phosphorylated by purified recombinant PKA (Fig. 2B).

To determine whether Ser988 is phosphorylated in vivo, α4 was metabolically labeled by [32P] in Jurkat T cells. The α4 subunit was isolated by immunoprecipitation followed by SDS-PAGE. Tryptic digests of the isolated subunit were subjected to two-dimensional phospho-peptide mapping. The phospho-peptide patterns were compared, with those observed with the α4 tail phosphorylated by PKA in vitro. A major co-migrating phospho-peptide spot, labeled 1 in Fig. 2C, was observed (PKA phosphorylated α4 in the left panel and metabolically labeled α4 in the right panel). Thus, the major tryptic α4 phosphopeptide isolated from Jurkat T cells is also present in PKA-phosphorylated α4. As noted above, α4 Ser988 is a predicted PKA phosphorylation site, and the major α4 phosphopeptide from Jurkat cells co-migrates with one from PKA-phosphorylated α4. These data suggest that α4 Ser988 is a major phosphorylation site in vivo. To test the importance of Ser988 phosphorylation, we assessed in vitro phosphorylation of an α4/S988A mutant by both PKA and a Jurkat cell lysate (Fig. 2D). This substitution completely abolished α4 phosphorylation by PKA and eliminated ∼90% of the α4 phosphorylation by the Jurkat cell lysate. Taken together, these results indicate that Ser988 is a major phosphorylation site in the α4 tail.

Regulation of Paxillin Binding by Phosphorylation of α4 Ser988—Ser988 resides in the middle of the paxillin binding sequence of the α4 tail (Fig. 1A). Consequently, we asked whether phosphorylation of Ser988 could regulate paxillin binding. We examined the binding of recombinant paxillin to α4 tail model proteins that had been phosphorylated in vitro with PKA to stoichiometries of 0.95 mol of PO4/3 mol of α4 tail. Phosphorylation of α4 abolished paxillin binding. Furthermore, paxillin binding could be reconstituted by dephosphorylation with alkaline phosphatase (Fig. 3A). Thus, PKA phosphorylation in vitro blocks the binding of the α4 tail to paxillin.
As noted above, α4 Ser<sup>388</sup>, the PKA phosphorylation site, is a major target of phosphorylation in Jurkat T cells. To determine whether phosphorylation of intact α4 regulated paxillin binding, we assessed the co-precipitation of α4 with paxillin. In previous studies (10), we found that nearly 100% αβ1 in Jurkat T cells could associate with paxillin when the cell lysates were prepared in the absence of phosphatase inhibitors. However, inclusion of phosphatase inhibitors resulted in a 2–3-fold decrease in α4 co-precipitated with paxillin (Fig. 3B). This decrease correlated with a 2–3-fold increase in α4 phosphorylation under these conditions (Fig. 3C). Thus paxillin binding to the α4 tail is regulated by phosphorylation of Ser<sup>388</sup>.

α4 Ser<sup>388</sup> Can Be Phosphorylated to High Stoichiometries in Vivo—The previous results suggested that α4 phosphorylation at Ser<sup>388</sup> could regulate paxillin binding. To evaluate the potential biological relevance of this observation, we assessed the stoichiometry of α4 phosphorylation. To quantitatively assay α4 phosphorylation, we raised a polyclonal antibody against an α4 synthetic peptide containing phosphoserine at Ser<sup>388</sup>. This antibody was rendered phospho-specific by absorption with non-phosphorylated α4 tail. The absorbed antibody (PSα4) failed to react with α4 tail but did so when the α4 tail was phosphorylated with PKA (Fig. 4A). To further assess the specificity of this antibody for phospho-α4, we also examined whether PSα4 recognizes a phosphorylated serine residue in the context of a similar flanking sequence. As shown in Fig. 4A (right panel), this antibody did not cross-react with a GST fusion protein phosphorylated in its PKA recognition site (RSASV). Furthermore, PSα4 reacted with phospho-α4 isolated from Jurkat T cells (Fig. 4B). Reactivity was abolished by dephosphorylation of the isolated α4 with alkaline phosphatase (Fig. 4B). Thus, the PSα4 specifically reacts with phosphorylated but not with non-phosphorylated α4 in a sequence-specific manner.

We used the PSα4 antibody to assess the stoichiometry of α4 phosphorylation. Preclearing cell lysates with PSα4 removed 60% of the total α4 from Jurkat T cells (Fig. 4C). In sharp contrast, when α4 was dephosphorylated by omission of phosphatase inhibitors, preclearing with PSα4 antibody did not deplete α4 (Fig. 4C). Phosphorylation of ~60% of α4 in Jurkat cells can account for the 2–3-fold reduction in paxillin-associated α4 when phosphatase inhibitors are present (see Fig. 3B). Consequently, at least 60% of α4 is constitutively phosphorylated in these Jurkat T cells, and phosphorylated α4 manifests reduced association with paxillin.

A Mutation That Mimics α4 Phosphorylation Disrupts Paxillin Binding and Promotes Cell Spreading—To further address the functional role of α4 phosphorylation at Ser<sup>388</sup>, we generated an α4 mutant that mimics constitutively phosphorylated status (S988D). To confirm that the aspartic acid substitution mimicked the biochemical effects of α4 phosphorylation, we examined the binding of recombinant paxillin to wild-type and mutant α4 tail model proteins. Aspartic acid substitution at Ser<sup>388</sup> markedly reduced paxillin binding (Fig. 5A). To assess a biological consequence of α4 phosphorylation, we examined the effect of this mutation on α4-dependent cell spreading. Paxillin binding to α4 cytoplasmic domain inhibits cell spreading, and therefore, disruption of this interaction results in increased cell spreading (10, 11). To examine the effect of the S988D mutation on cell spreading, we transiently transfected Chinese hamster ovary cells with either α4 or α4(S988D). Cells transfected with α4(S988D) spread promptly on the CS-1 fragment of fibronectin, a ligand of integrin αβ1 (Fig. 5, B and C). In sharp contrast, cell spreading was markedly retarded in cells expressing the wild type α4 (Fig. 5, B and C). Thus, phosphorylation of α4<sup>S988</sup> leads to loss of paxillin binding with consequent alterations in cellular response to α4β1-mediated adhesion.

In this study, we found that integrin α4 is phosphorylated on a serine residue(s) and that Ser<sup>388</sup> is a major target of phosphorylation. Phospho-peptide mapping showed that the major α4 phospho-peptide was that containing Ser<sup>388</sup>. Furthermore, the S988A mutation abolished ~90% of α4 phosphorylation by Jurkat cell lysate. The tryptic phospho-peptide contains two other potential phosphorylation sites (Ser<sup>3990</sup> and Ser<sup>3994</sup>). However, immunoprecipitation and immunoblotting with PSα4 antibody verified the phosphorylation on Ser<sup>388</sup>. Consequently, Ser<sup>388</sup> is a major site of phosphorylation. Our data show that this site is used by PKA in vitro. However, we emphasize that this site could be a target of other kinases (e.g., Ca<sup>2+</sup>-calmodulin-dependent protein kinase II, protein kinase G) in vivo. The development of the Ser<sup>388</sup> phospho-specific antibody should facilitate the identification of the relevant kinases.

Our studies identify α4 phosphorylation at Ser<sup>388</sup> as a potential regulator of α4 integrin signaling. In particular, the interaction of α4 with paxillin (or one of its paralogs) is required for α4 specific promotion of cell migration, enhanced phosphorylation of pp125FAK (focal adhesion kinase (FAK)) and reduction of cell spreading (10). Phosphorylation at Ser<sup>388</sup> abolishes this interaction. Consequently, the phosphorylation will modulate all of the responses dependent on the α4-paxillin interaction. Previous studies report regulatory phosphorylation of integrin tails. For example, Tyr phosphorylation of β3 and β1 tails regulates integrin signaling and migration (23, 24). The effects of β3 Tyr phosphorylation may be because of altered interactions with either myosin or the SHC adaptor (25, 26). In addition, β3 phosphorylation at Thr<sup>753</sup> has been proposed to regulate bi-directional integrin signaling (27, 28). α1 and αM can be phosphorylated on their serine residues (29–31), and these phosphorylations may regulate cytoskeletal associations. Furthermore, α4 and α7 Ser phosphorylation may regulate cell shape and cell migration (32, 33). However, the effects of these α subunit phosphorylations on specific biochemical interactions of the α tails have not been defined. In the present study, we have identified a high stoichiometry phosphorylation of an integrin α tail that regulates a protein-protein interaction that controls biological responses. Temporal and spatial regulation of α4 phosphorylation may thus be an important biochemical mechanism for the control of α4-mediated cell functions.

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Serine Phosphorylation of α₄ Integrin

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Phosphorylation of the Integrin α₄ Cytoplasmic Domain Regulates Paxillin Binding
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