Tyrosine Phosphorylation as a Conformational Switch

A CASE STUDY OF INTEGRIN \( \beta_3 \) CYTOPLASMIC TAIL

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Reversible protein phosphorylation is vital for many fundamental cellular processes. The actual impact of adding and removing phosphate group(s) is 3-fold: changes in the local/global geometry, alterations in the electrostatic potential and, as the result of both, modified protein-target interactions. Here we present a comprehensive structural investigation of the effects of phosphorylation on the conformational as well as functional states of a crucial cell surface receptor, \( \alpha_{\text{IIb}} \beta_3 \) integrin. We have analyzed phosphorylated (Tyr\(^{747} \) and Tyr\(^{759} \)) \( \beta_3 \) integrin cytoplasmic tail (CT) primarily by NMR, and our data demonstrate that under both aqueous and membrane-mimetic conditions, phosphorylation causes substantial conformational rearrangements. These changes originate from novel ionic interactions and revised phospholipid binding. Under aqueous conditions, the critical Tyr\(^{747} \) phosphorylation prevents \( \beta_3 \)CT from binding to its heterodimer partner \( \alpha_{\text{IIb}} \)CT, thus likely maintaining an activated state of the receptor. This conclusion was tested in vivo and confirmed by integrin-dependent endothelial cells adhesion assay. Under membrane-mimetic conditions, phosphorylation results in a modified membrane embedding characterized by significant changes in the secondary structure pattern and the overall fold of \( \beta_3 \)CT. Collectively these data provide unique molecular insights into multiple regulatory roles of phosphorylation.

Protein phosphorylation, initially discovered in the mid 1950s (1), today is considered as one of the most crucial cell signaling events. It is a reversible, ubiquitous switch which regulates nearly every aspect of prokaryotic and eukaryotic cell life and has been linked to many pathogenic processes. Phosphorylation involves a covalent attachment of the negatively charged phosphate group to the side chains of serine (86.4%), threonine (11.8%), and tyrosine (1.8%) residues in eukaryotes (2), which may result in local and/or global conformational rearrangement or induce transitions from order to disorder and vice versa (3). Moreover, it may alter protein function by modifying its interactions with the substrates or by varying the equilibrium between different conformational states. A total comprehension of these transitions is crucial for enhancing our knowledge of the signal transduction processes.

Integrins, a major class of non-covalent heterodimeric glycoprotein cell surface receptors, have been chosen for investigating the effects of phosphorylation in present work. Integrins are among the most studied and best characterized cell adhesion molecules. Each integrin subunit contains a large extracellular ligand-binding portion, a single membrane-spanning domain, and a short cytoplasmic tail devoid of any enzymatic activity (4). The unique bidirectional flow of information through integrins involves inside-out signals, which allow them to interact with extracellular soluble ligands, and ligand-dependent outside-in signals, which trigger the cellular response to cell adhesion. The integrins extracellular matrix (ECM)\(^2\) interactions are controlled by integrins extracellular domains, whereas integrin-cytoskeletal proteins interactions are controlled via their cytoplasmic tails (CTs). This integration of extracellular and intracellular compartments allows dynamic regulation of many cellular processes including cell migration, shape change, proliferation, and differentiation (5). Integrin regulated signaling pathways, which involve direct or indirect interaction of the integrin CTs with integrin-associated proteins, are often controlled through phosphorylation.

Although platelet integrin \( \beta_3 \)CT includes several phosphorylation sites (two tyrosines, one serine and multiple threonines), only tyrosine phosphorylation is found to be specific for the outside-in signaling (6). However, despite the crucial role of tyrosine phosphorylation for \( \beta_3 \) integrin function, the structural details describing the consequences of this process remain unknown. In this study we have investigated the effects of tyrosine phosphorylation on \( \beta_3 \)CT under both, aqueous and membrane-mimic, environments. Our data demonstrate that, in comparison to the non-phosphorylated form (7, 8), under aqueous conditions phosphorylation of Tyr\(^{747} \) and/or Tyr\(^{759} \) of \( \beta_3 \)CT induces a novel fold which precludes \( \alpha_{\text{IIb}} \beta_3 \) complex formation, thereby preserving the activated state of the recep-

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\(^2\)The abbreviations used are: ECM, extracellular matrix; Ab, antibody; \( \beta_3 \)NP, non-phosphorylated \( \beta_3 \)CT; CT, cytoplasmic tail; DPC, dodecyl-phosphocholine; DSA, doxyl stearic acid; FN, fibronectin; IPTG, isopropyl \( \beta \)-D-1-thiogalactopyranoside; KO, knock-out; K\textsubscript{sv}, Stern-Volmer quenching constant; MS, mass spectrometry; NOESY, nuclear overhauser enhancement spectroscopy; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum correlation; PR\(_E\), paramagnetic relaxation enhancement; RP-HPLC, reversed phase high pressure liquid chromatography; VN, vitronectin.
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In presence of dodecyl-phosphocholine (DPC) tyrosine(s) phosphorylation results in significant conformational rearrangements of $\beta_3$CT coupled to a considerable perturbation of its interaction with the membrane. Together, these data define a critical role of tyrosine phosphorylation, in general, in the regulation of signal transduction as well as in controlling $\beta_3$-integrin function.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Short tyrosine(s)-phosphorylated peptides corresponding to NMP, BP, and BP-Pep (729THDrKE-FAKKFEERARKWDTANNLPKYK) and (729RAKWD-TANNLPYKEATSTFTNTpYRGT) respectively, were chemically synthesized (Genemed Synthesis, Inc.). Expression and Purification—Cloning, expression, and purification of $\alpha_{IIb}$CT, $\beta_3$CT (non-phosphorylated form hereafter referred to as $\beta_3$NP), and MP-$\alpha_{IIb}$ have been described previously (7). To produce $^{15}$N and/or $^{13}$C isotopically labeled $\alpha_{IIb}$CT and $\beta_3$CTs, cells were grown in M9 minimal medium containing $^{15}$NH$_4$Cl (1.1 g/liter) and $^{13}$C-glucose (2.5 g/liter) as the sole source of nitrogen and carbon. Tyrosine phosphorylation of $\beta_3$CT (mono-phosphorylated at Tyr-747, hereafter referred to as $\beta_3$MP, and bi-phosphorylated at Tyr-747 and Tyr-759, hereafter referred to as $\beta_3$BP) has been achieved in vivo by using TKB1 bacterial cell line from Stratagene following the manufacturer’s protocol for the recombinant protein induction. Single amino acid mutations were made by using the QuikChange site-directed mutagenesis kit (Stratagene).

**Mass Spectroscopy**—Mass spectral analyses were performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR Elite) equipped with an ESI source. The data acquisition was under the control of the Analyst QS software (Foster City, CA). All samples were dissolved in methanol:water mixture to achieve final concentration of 40 $\mu$M. Samples were infused into the ESI source at a flow rate of 10 $\mu$l/min by using the built-in syringe pump. Typical source conditions for Q-STAR were as follows: capillary voltage (5500V), declustering potential (215V), resolution (15000, full width-half maximum).

**Tryptophan Fluorescence Quenching**—Steady-state fluorescence was measured with a SPEX Fluorolog FL3–22 spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. Emission scans for Trp fluorescence ($\lambda_{em} = 295$ nm; $\lambda_{ex} = 310$ to 400 nm at 1-nm intervals with 2 nm and 4 nm excitation and emission bandwidth, respectively) were performed using samples (20–37 $\mu$M protein, pH 5.9) in 4 x 4 mm quartz microcells. Iodide Trp quenching was measured by titrating two equivalent samples in parallel with aliquots from either 2.5 M KI or 2.5 M KCl stocks, each containing 5 mM Na$_2$S$_2$O$_3$. Maximum intensities from each KI titration point were corrected by the KCl-containing samples to achieve the sole source of nitrogen and carbon. Tyrosine phosphorylation was measured by titrating two equivalent samples in parallel containing 5 mM Na$_2$S$_2$O$_3$. Maximum intensities from each KI titration point were corrected by the KCl-containing samples to achieve final concentration of 40 $\mu$M. Samples were infused into the ESI source at a flow rate of 10 $\mu$l/min by using the built-in syringe pump. Typical source conditions for Q-STAR were as follows: capillary voltage (5500V), declustering potential (215V), resolution (15000, full width-half maximum).

**EC Transfection and Adhesion Assay**—Lung EC at passage 2 were suspended at 5 x 10$^6$/ml in OptiMEM media with 10 $\mu$g/ml pCDNA3.1 (empty or expressing $\beta_3$WT or mutants) and 2 $\mu$g/ml pMAX-GFP. 100-$\mu$l portions were transferred to nucleofection cuvettes and pulsed using amaxa Biosystems nucleofector, program M-003. The cells were plated in growth media on VN-coated dishes, and sodium butyrate (at 5 mM final concentration) was added. 96-well plates were coated 1 h at 37°C with 0.1 $\mu$g/ml VN, blocked 1 h at 37°C with 1% heat-denatured BSA in PBS and washed in 3 x PBS. Transfected cells were harvested 72 h post-transfection by brief trypsinization, washed, and suspended in DMEM:F12 containing 0.2% BSA. 2 x 10$^4$ cells in 60 $\mu$l volume were plated per well and allowed to adhere for 25 min. The wells were washed 5 x in DMEM:F12 and the cells fixed 10’ in 2% formaldehyde in PBS. GFP images at 5x magnification were acquired and composite images encompassing the entire wells were constructed. Amounts of GFP-positive cells per well were quantified using ImageJ software. Portion of the transfected cells were analyzed by FACS using $\alpha_{IIb}$, $\beta_3$ antibodies to compare expression levels of the $\beta_3$ constructs.

**NMR Spectroscopy**—Chemical shift assignments of $\beta_3$CT have been determined previously (7) and have been modified to address the effects of phosphorylation and changes in pH values. All the NMR experiments were performed on Varian 600MHz and 800 MHz equipped with inverse-triple resonance cold-probes and were processed with NMRpipe (9) and analyzed by CCPN software suite (10). Earlier we have used aqueous conditions (pH 6.1) to understand the $\alpha_{IIb}$ heterodimer interface (7). However, because of the apparent solubility issues of phosphorylated constructs at pH 6.1, pH of the $\beta_3$MP samples was reduced to 5.9 to achieve sufficient concentrations for structure determination. Both $\beta_3$NP and $\beta_3$MP did not demonstrate substantial pH-dependent conformational differences judging by their chemical shift perturbation data (supplemental Fig. S4A).$^3$H-$^1$H HSQC titration experiments (Fig. 3, C and D) were performed in water at 25°C at pH 6.1. Transferred NOESY experiments (Fig. 3, E and F) for different peptides were performed at 25°C at pH 6.1. Different ratios of the peptides to the binding partner were investigated to find the optimal range for NOE transfer for each particular analysis. The resonance assignments of unlabeled peptides were made using conventional two-dimensional $^1$H–$^1$H TOCSY and NOESY spectra (11) by CCPN software suite (10).

To characterize the structures and membrane-binding properties of phosphorylated $\beta_3$CTs, 0.07–0.9 mM $^{15}$N- and/or $^{13}$C-labeled $\beta_3$CTs ($\beta_3$NP, $\beta_3$MP and $\beta_3$BP) were dissolved in 60–300 mM deuterated DPC solution (Sigma-Aldrich) prepared in 20 mM sodium phosphate buffer, 5 mM Ca$^{2+}$ at pH 5.9. The pH was monitored with pH strips (EMD Chemicals). All NMR experiments involving membrane-mimetic conditions were performed at 40°C. To determine the location of different $\beta_3$ constructs relative to the micelle surface, steric acid compounds (16-DSA and 5-DSA) were used. Both were dissolved in 50 mM deuterated DPC solution to make 50 mM stock solution. These solutions were then added to the protein + DPC solution to achieve the following final ratios of protein: 5/16 DSA; DPC: 1:10:750 ($\beta_3$NP); 1:12:1000 ($\beta_3$MP); 1:14:1000 ($\beta_3$BP). The effects of the spin labels were observed by comparing the peak intensities (supplemental Fig. S6) in $^3$H-$^1$N HSQC spectra. For
calculating the intensity ratios, the spectra were processed with 10 Hz exponential broadening in direct dimension and zero-filled to 2048 × 1024 data points in t2 and t1, respectively. For the NMR dynamics study of β3NP, 1H-15N NOE, 13N T1, and T2 data under aqueous (25 °C) and membrane mimetic conditions (40 °C) were collected on a Varian Inova 600 MHz spectrometer. 15N T1 values were measured from the spectra recorded with 8 different durations of the delay: T = 30, 90, 150, 250, 400, 600, 800, 1200 ms. 15N T2 values were determined from spectra recorded with 8 different durations of the delay: T = 10, 20, 30, 50, 70, 90, 110, 150 ms. Steady-state hetero-nuclear 1H-15N NOE values were determined from spectra recorded with 5 s relaxation delay and the presence and absence of a proton presaturation period of 5 s. T1, T2, and NOE values were extracted by a curve-fitting subroutine included in the CCPN software suite (10) (supplemental Fig. S7). The rotational correlation time (τR) values were estimated to be ~5 ns in aqueous solution and 9 nanoseconds in DPC using TENSOR-2 (32) indicating the differences in overall tumbling associated with micelles binding.

Structure Calculation—Table 1 lists detailed structural statistics of the final fifteen lowest energy conformers of β3NP, β3MP, and β3BP under aqueous and membrane-mimetic conditions along with the two-dimensional and three-dimensional NMR experiments utilized for individual structure determination. For β3MP and β3BP in presence of DPC micelles, the backbone, ϕ, and ψ, dihedral angle restraints were obtained by using Talos+ (12). All the initial structure calculations were performed using CYANA 2.1 (13). Hydrogen bond restraints (in the case of β3MP under aqueous conditions) were introduced during the final stages of calculations. Sixty lowest energy structures from CYANA were subjected to molecular dynamics simulations in explicit water (14) using CNS (15). For β3NP under membrane-mimetic conditions, sixty structures were calculated by utilizing previously (8) determined NOE and dihedral restraints with the help of CYANA and later were refined in explicit water to maintain consistency and for a more accurate comparison. None of the structures have NOE and dihedral angle violations more than 0.5 Å and 5°, respectively. The Protein Structure Software suite (PSVS; courtesy of CABM Structural Bioinformatics Laboratory, Rutgers State University of New Jersey) was used for structure quality assessment and validation.

RESULTS

Preparation of Tyrosine-phosphorylated Integrin β3 Cytoplasmic Tail for Structural Analysis—Phosphorylation has long been considered as a critical regulatory apparatus in signal transduction and nuclear magnetic resonance (NMR) spectroscopy is a pertinent technique for deciphering the emanating conformational changes imparted by phosphorylation. However, the first step in investigation of phosphorylation by NMR, production of the phosphorylated, isotopically labeled proteins in adequate amounts, is usually an uphill task. In the case of β3CT (see supplemental Fig. S1 for sequence details), the TKB1 bacterial cell line (Stratagene), carrying Elk tyrosine kinase gene controlled by the trp promoter, was found to yield sufficient quantities of (tyrosine)-phosphorylated protein. Although it has been suggested that this in vivo approach is not as efficient as the in vitro technique due to the deleterious effects of Elk tyrosine kinase on the bacteria (16), we could produce reasonable amounts of phosphorylated β3 by using unusually short induction times (IPTG induction of β3 at A600 ~0.3 followed in 2 h by tryptophan induction of Elk). Supplemental Fig. S2 (SM) depicts the reversed phase, RP-HPLC chromatogram of β3CT expressed in TKB1 cells and the deconvoluted mass spectra of the three HPLC peaks. The MS analysis reveals that the peaks eluting at 23, 24, and 26% of acetonitrile gradient correspond to the bi-phosphorylated, mono-phosphorylated, and non-phosphorylated β3CT, respectively. Our initial assumption was that we will achieve almost equal populations of the two mono-phosphorylated β3CT constructs (pY747 and pY759) respectively which would be very difficult to separate. However, a closer inspection of superimposed 1H-15N HSQC spectra of these three HPLC peaks, Fig. 1A and supplemental Fig. S3A, demonstrates that the middle peak in the chromatogram is pY747-β3CT and does not contain any pY759-β3CT. Though Elk is known to be a promiscuous tyrosine kinase, the efficiency of phosphorylation in β3CT appeared to be very different for the two tyrosine residues (Ty747, Ty759). While we could produce NMR quantities of pY747-β3CT (hereafter referred to as β3MP) and extremely limited quantities of pY747, pY759-β3CT (hereafter referred to as β3BP); we could not generate any pY759-β3CT. One possible explanation for the lack of pY759 product may be the flexibility and dynamic nature of the β3CT’s C terminus in the absence of pY747. To substantiate this hypothesis, we introduced conservative point Y747F or Y759F mutations in β3CT construct by using site-directed mutagenesis (QuikChange). Supplemental Fig. S3B depicts the superimposition of 1H-15N HSQC spectra of these mutants with the wild type β3CT. Expression of these mutants in TKB1 cells confirmed our observation. As in the case of their wild type counterparts, we could produce pY747 for Y759F mutant (supplemental Fig. S3C), but were unable to phosphorylate the Y747F mutant.

Phosphorylation of Tyr747 Results in Structural Rearrangement of β3CT under Aqueous Conditions—The superimposition of 1H-15N-HSQC spectra for β3MP, β3MP, and β3BP under aqueous conditions is shown in Fig. 1A and the subsequent chemical shift perturbations are presented in Fig. 1C. From these data, it is clear that phosphorylation not only affects the nearby residues (743NN-LpYKEA750 and 757ITpYRGT762) but also influences the membrane-proximal region (K716-D723). These latter chemical shift perturbations are common to both, β3MP and β3BP constructs, suggesting an intramolecular interaction between the membrane-proximal and 743NLPY747 regions. This interaction is a direct result of Tyr747 phosphorylation, most probably due to a formation of the salt bridge between the negatively charged phosphate group and the positively charged/polar side-chain(s) of the N-terminal amino acid(s). To better understand this change, we acquired 1H-13N two dimensional (2D) Nuclear Overhauser Enhancement Spectroscopy (NOESY) and three-dimensional 15N-edited NOESY spectra of β3MP, which allowed us to structurally characterize β3MP under aqueous conditions. The overall fold of β3MP is shown in Fig. 2, A and B (PDB ID: 2jfl, see statistics in Table 1). Interestingly, the negatively charged phosphate group of pY747

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creates a salt-bridge with the positively charged side-chain of Lys738, which, in turn, affects the orientation of Trp739 indole side chain. This bulky, hydrophobic side chain forges contacts with the methyl groups of membrane-proximal residues (Leu717 is shown as an example, Fig. 2B) and the resulting compact conformation is then further stabilized by hydrogen bond between the side-chains of D740-N743. The first turn of the membrane-proximal helix, found in the αhelβ3 heterodimer (7), is not formed in β3MP and the helical region spans only from Lys725 to Lys729. The C-terminal region (F754-T762) is dynamically unstructured. Interaction of Trp739 with the membrane-proximal residues supports the notion that it could be situated near, but not necessarily within, the membrane. This is consistent with our prior findings that both Trp739 and Tyr747 can interact with phospholipids (8). Compared with the compact conformation of β3MP, β3NP is much more dynamic and largely unstructured, as has been reported previously (7, 17), except for a reverse turn formed around the 744NPLY747 motif and helical tendencies in the membrane proximal region (Fig. 2, E and F, statistics of the ensemble are presented in Table 1). Moreover, the dynamic nature of β3NP under aqueous conditions is supported by NMR relaxation measurements as most of the HetNOE values are below 0.3 (supplemental Fig. S7, A–D). Sequential connectivity maps for both ensembles are provided (supplemental Fig. S5).

It is imperative to mention that in the case of β3MP, although the chemical shift changes due to pY747 are very modest and absolute chemical shift values are close to the random coil values, we were able to observe long-range NOEs. In dynamic systems where the folded, transient, and unstructured conformers are in fast exchange, the observed NOEs can be averaged over multiple conformers, and not all the contacts are satisfied by a single conformer. Any attempt to fulfill all such NOE contacts simultaneously might lead to an over-constrained system. Hence, to independently confirm this novel compact conformation of β3MP is not artificial, we performed iodide quenching of tryptophan fluorescence to determine the relative exposure of W739 indole side chain of β3MP and β3NP to the aqueous environment (Fig. 2C). The measured KSV values were lower for β3MP (KSV = 3.09 ± 0.43 M−1) than for β3NP (KSV = 4.35 ± 0.56 M−1). Our steady-state emission scans strongly suggest that the higher KSV values of the β3NP are not due to the highly unquenched fluorescence lifetime because the specific fluorescence intensities and λmax values are equivalent for both proteins. This indicates that Trp739 in β3MP is indeed better shielded from solvent exposure than its non-phosphorylated counterpart by virtue of its compact fold and due to the hydrophobic interactions with the N-terminal residues. In addition, we investigated the importance of pY747-K738 salt bridge by using NMR salt titrations. The rationale behind these experiments was that the electrostatic interactions responsible for the β3MP fold should be affected by changes in salt concentrations. Supplemental Fig. S4B displays the distribution of chemical shifts for β3MP under the different ionic strength buffers. High salt concentration, indeed, has a major impact on the changes in chemical shifts resulting in significant decline of chemical shift perturbations throughout the membrane proximal region of β3CT.

To further validate and test the biological significance of this fold, we performed site-directed mutagenesis, point mutation K738E (Quikchange) (see supplemental Fig. S1 for sequence details) and investigated both in vitro and in vivo properties of

**FIGURE 1. Tyrosine(s) phosphorylation of β3 CT.** 1H-15N HSQC spectra of 15N-labeled β3NP (black), β3MP (red), and β3BP (lime): (A) in aqueous solution, pH 6.1, 25 °C; (B) in DPC micelles, pH 5.9, 40 °C. Some critical residues undergoing conformational change are labeled and marked with arrows. Chemical shift changes of β3 tails due to tyrosine phosphorylation: (C) in aqueous solution, pH 6.1, 25 °C; (D) in DPC micelles, pH 5.9, 40 °C. Delta [ppm] refers to the chemical shift changes according to the equation: ΔS(HN,N) = (∆δHN + 0.2(∆δN)2)1/2, where ΔS = S(hound − S)exp. Bars are colored as follows: β3NP versus β3MP (dark gray) and β3NP versus β3BP (light gray).
this mutant. Residue Lys$^{738}$ was selected for this analysis as it plays a critical role in the conformation change of β$_3$MP and as per our knowledge has not been implicated in any interactions with known integrin modulators, such as talins or kindlins. Hence the effect of this mutation on integrin activation state should be attributed to the structural integrity rather than external factors. Theoretically, this K738E charge reversal should abolish the salt-bridge in β$_3$MP, making the conformational change due to Tyr$^{747}$ phosphorylation challenging. As expected, the chemical shift perturbations in the membrane proximal region due to Tyr$^{747}$ phosphorylation are smaller than its wild type (WT) counterpart and are randomly distributed.
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TABLE 1

|                     | β3NP (water) | β3MP (water) | β3NP (DPC) | β3MP (DPC) | β3BP (DPC) |
|---------------------|--------------|--------------|------------|------------|------------|
| Distance restraints |              |              |            |            |            |
| All                 | 797          | 798          | 925        | 603        | 505        |
| Short range (i−j<1) | 482          | 293          | 703        | 436        | 374        |
| Medium-range (1<i−j<5) | 314        | 440          | 202        | 147        | 121        |
| Long-range (i−j>5)  | 1            | 65           | 20         | 20         | 10         |
| Ambiguous           | 7            | 10           | NA         | NA         | NA         |
| Dihedral Angle restraints× |
| Phi (d)              | NA           | NA           | 33         | 28         | 29         |
| Psi (d)              | NA           | NA           | 32         | 28         | 29         |
| Hydrogen bond restraints× |
|                         | NA           | 2            | NA         | NA         | NA         |
| Average CYANA target function value | 2.80 | 3.71 | 2.54 | 1.42 | 0.77 |

V operator = 0.0318 ± 0.0015 | NOE
vdw | 0.0329 ± 0.0013 |
vdw | 0.0261 ± 0.0014 |
vdw | 0.0261 ± 0.0012 |
vdw | 0.0260 ± 0.0016 |

RMSD =
Average backbone RMSD to mean | 2.08 Å
Average heavy atom RMSD to mean | 3.18 Å

VanderWaal Energy (kcal mol−1)× |
| −394.26 ± 8.52 | −399.48 ± 33.17 | −414.26 ± 15.52 | −373.72 ± 27.84 | −367.34 ± 27.40 |

Deviation from idealized geometry |
| Bonds (Å) | 0.0134 ± 0.00056 | 0.0145 ± 0.00020 |
| Angles (°) | 1.3771 ± 0.0363 | 1.4163 ± 0.0449 |
| Improper (°) | 1.2315 ± 0.1380 | 1.5317 ± 0.1282 |

Ramachandran statistics× |
| Residues in favored regions | 72.6% | 49.3% | 85.4% | 78.3% | 82.2% |
| Residues in allowed regions | 23.6% | 46.5% | 9.9% | 18.3% | 16.6% |
| Residues in generously allowed regions | 1.7% | 3.9% | 3.4% | 3.0% | 1.1% |
| Residues in disallowed regions | 2.2% | 0.3% | 1.9% | 0% | 0.2% |

NMR experiments |
| 2D 1H-1H NOESY | 3D 1H-1H NOESY | 3D 15N-edited NOESY × Based on Ref. 8 |
| 3D 13C-edited NOESY | 3D 13C-edited aromatic NOESY | 3D 13N-edited NOESY | 3D 1H NOCO | 3D HNCO |
| 3D HNCA/CB |

× Generated from Talos ± (12).
× Hydrogen bonds were introduced in the last stage of structure calculations.
× The following residues are considered for the rmsd calculations: I) β3NP (water): residues 720–735 II) β3MP (water): residues 720–745 III) β3NP (DPC): residues 722–745 IV) β3MP (DPC): residues 720–745 V) β3BP (DPC): residues 720–745.
× After refinement in explicit water by using CNS(15).
× All residues, calculated using the Protein Structure Software suite.
× 400 ms mixing time.
× 300 ms mixing time.
× 150 ms mixing time.

throughout the sequence with the major local effect shifted in C-terminal direction (supplemental Fig. S4C). Thus it can be argued that under in vitro conditions β3NP K738E mutant probably exhibits behavior similar to that of β3NP. The effect of this charge reversal was next tested in β3 integrin-dependent endothelial cell (EC) adhesion to vitronectin (VN). EC isolated from β3 knock-out (KO) mice were transfected with expression vectors for either WT β3 or substitution mutants Y747F, K738E, Y747F-K738E (see supplemental Fig. S1 for sequence details). As shown in Fig. 2D, expression of both K738E and Y747F mutants showed decreased adhesive response as compared with WT β3 integrin expressing cells. Importantly, adhesion of K738E-expressing cells was similar to that of Y747F mutant and no further inhibition was observed in double K738E/Y747F mutant cells. As an additional control, K738E mutation was tested to find out whether it can reduce integrin ability to undergo activation, for example by preventing its interactions with talins or kindlins. As expected, this mutation did not result in diminished soluble fibrinogen binding mediated by α<sub>IIbβ3</sub> integrin (supplemental Fig. S4D). Thus, the resulting differences in the activation states between this mutant and the wild type are related to the internal structural integrity. Combined, these data confirm the critical role of K<sup>738</sup>/Y<sup>747</sup> for the regulation of integrin-mediated cell adhesion and biological significance of novel β3 MP conformation.

Because of the challenges with sample preparation, we could not investigate structural details of β3BP under aqueous conditions, but considering the similarity of chemical shifts of N-terminal residues between β3 NP and BP (Fig. 1, A and C) and the dynamic nature of the C terminus, it is safe to suggest that β3BP accommodates a conformation similar to β3NP and is distinct from β3NP. Together these data indicate that upon phosphorylation β3CT undergoes a substantial structural change, leading to a more compact conformation, which, in turn, might affect interactions between the receptor and intracellular adaptors.

Tyrosine Phosphorylation Preserves the Activated State of Integrin by Preventing the Interaction between β3 and α<sub>IIb</sub>Cytoplasmic Tails—In a previous study (7), we have structurally characterized the cytoplasmic domain of α<sub>IIbβ3</sub> heterodimer. Our data revealed the underlying mechanism by which the inter-subunit clasps R<sup>995</sup>(α<sub>IIb</sub>CT)-D<sup>724</sup>(β3CT) along with several other electrostatic and hydrophobic contacts (Fig. 3A), maintains the integrin in a resting state. Termination of these interactions eventually results in integrin activation. As tyrosine phosphorylation leads to a conformational rearrangement of β3CT, we were curious to find out whether these changes
affect the formation of α<sub>IIb</sub>β<sub>3</sub> heterodimer. Superimposition of β<sub>3</sub>MP structure with α<sub>IIb</sub>β<sub>3</sub>3 heterodimer revealed a steric clash between β<sub>3</sub>MP and the α<sub>IIb</sub> subunit indicating the possible difficulties in clasp formation (Figs. 3B and 5A). To test this prediction, we performed chemical shift mapping experiments similar to those done before to define α<sub>IIb</sub>β<sub>3</sub>3 cytoplasmic clasp (7). Non-labeled β<sub>3</sub>NP, β<sub>3</sub>MP, and β<sub>3</sub>BP were titrated with 15N-labeled α<sub>IIb</sub>CT and the associated chemical shift perturbations were monitored. Chemical shift changes plotted as a function of residue numbers in α<sub>IIb</sub>CT (Fig. 3C) indicate a gradual decrease upon β<sub>3</sub>CT phosphorylation. These observations were also supported by the opposite experiments, where non-labeled α<sub>IIb</sub>CT is titrated into solutions of 15N-labeled β<sub>3</sub>NP, β<sub>3</sub>MP, and β<sub>3</sub>BP. Fig. 3D shows the subsequent chemical shift changes plotted as a function of the residue number in β<sub>3</sub>CT. It is important to mention that residues Lys<sup>748</sup> and Glu<sup>749</sup> are more perturbed in β<sub>3</sub>MP than in β<sub>3</sub>NP. This could indicate either the appearance of a new binding site, or an internal conformational rearrangement in β<sub>3</sub> tail. To examine the possibility of interaction between α<sub>IIb</sub>CT and residues Lys<sup>748</sup> and Glu<sup>749</sup> of β<sub>3</sub>CT, we utilized the transferred NOE (trNOE) method (18). This approach is well suited for characterization of such weak interactions and has been used in the elucidation of α<sub>IIb</sub>β<sub>3</sub> structure (7). The method detects appearance of additional peaks in the ligand’s NOESY spectra upon its interaction with the target protein (19). α<sub>IIb</sub>CT was fused to maltose-binding protein (MBP) tag to increase the molecular weight as higher molecular weight allows more favorable NOE transfer, the effect proven experimentally even despite of some independent local α<sub>IIb</sub>CT motion (7). Because the full-length β<sub>3</sub> tail has limited solubility (20), we have used shorter β<sub>3</sub> peptides, NMP<sub>β<sub>3</sub></sub> containing the N-terminal residues, including pY<sup>747</sup> and BP<sub>β<sub>3</sub></sub>Pep containing the C-terminal residues, including both pY<sup>747</sup> and pY<sup>759</sup> (see supplemental Fig. S1, SM, for sequence details). No additional peaks were detected in NOESY spectra of these peptides upon addition of MBP-α<sub>IIb</sub> under any of the conditions tested (Fig. 3, E and F). Thus based on all these data, it can be concluded that the chemical shift perturbations observed for residues Lys<sup>748</sup> and Glu<sup>749</sup>
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and Glu\textsuperscript{749} are due to an internal structural rearrangement. And tyrosine phosphorylation of β\textsubscript{3}CT, indeed, prevents β\textsubscript{3} from making and/or maintaining contacts with α\textsubscript{IIb}CT, thereby preserving the activated state of the receptor.

Phosphorylation Affects β\textsubscript{3}CT Interaction with the Membrane—
To address the effects of tyrosine(s) phosphorylation on β\textsubscript{3}CT’s interaction with lipid bilayer, we next investigated β\textsubscript{3}MP and β\textsubscript{3}BP in DPC detergent micelles. DPC has been used extensively as a membrane mimetic for NMR studies and was previously utilized to structurally characterize β\textsubscript{3}NP (8) and other similar constructs (21, 22). In the presence of DPC micelles, β\textsubscript{3}NP exhibits much more structured conformation even without its binding partner α\textsubscript{IIb}CT (8). Moreover, we have demonstrated that several residues of β\textsubscript{3}NP (Trp\textsuperscript{739}, Thr\textsuperscript{741}, Ala\textsuperscript{742}, Pro\textsuperscript{745}, and Tyr\textsuperscript{747}) could interact with DPC micelles and these interactions initiate the formation of a second short α-helical region (Leu\textsuperscript{746}, Asn\textsuperscript{748}), which is not generally observed in either aqueous β\textsubscript{3} or α\textsubscript{IIb}β\textsubscript{3} heterodimer. These conclusions are corroborated by the NMR relaxation measurements (supplemental Fig. S7, E–H). The whole β\textsubscript{3}NP is tumbling along with the micelles except the last three C-terminal residues (RGT\textsuperscript{763}) which are undergoing a significant local motion. However, both β\textsubscript{3}MP and β\textsubscript{3}BP behaved very differently as compared with β\textsubscript{3}NP in DPC micelles. The superimposition of \textsuperscript{1}H-\textsuperscript{15}N-HSQC spectra of β\textsubscript{3}NP/MP/BP in DPC is depicted in Fig. 1B and the resultant chemical shift perturbations are presented in Fig. 1D. The shift differences between non-phosphorylated and phosphorylated constructs are quite significant (Fig. 1D). This may be due to the alterations in secondary structural elements because of the distinctive β\textsubscript{3}-membrane interaction. Interestingly, the chemical shifts are almost identical for the affected mid-region (residues E\textsuperscript{731}-T\textsuperscript{753}) in β\textsubscript{3}MP and β\textsubscript{3}BP cases, indicating the likeness of the conformations in membrane environment. For β\textsubscript{3}BP, as expected, the additional shift changes were associated with the second phosphorylation site (the C terminus residues, F\textsuperscript{754}-T\textsuperscript{762}) highlighting the possible differences from β\textsubscript{3}MP. To confirm these hypotheses, we have performed a full scale NMR structural investigation of β\textsubscript{3}MP (and partially for β\textsubscript{3}BP) in DPC micelles. We have also tested the interactions of all three constructs (β\textsubscript{3}NP/MP/BP) with the DPC micelles using the Paramagnetic Relaxation Enhancement (PRE) approach (23).

The overall folds of β\textsubscript{3}NP, β\textsubscript{3}MP and β\textsubscript{3}BP are shown in Fig. 4, A, C, E, respectively (PDB IDs: 2jdj and 2jle for β\textsubscript{3}MP and β\textsubscript{3}BP, respectively. The statistics are shown in Table 1; see under “Experimental Procedures” for additional details. Sequential connectivity maps are provided in supplemental Fig. S5). The prominent structural features of β\textsubscript{3}NP in presence of DPC micelles, a membrane-proximal α-helix (K\textsuperscript{716}-R\textsuperscript{734}) followed by a flexible loop and another short helix (Y\textsuperscript{737}, T\textsuperscript{759}) (8), are extensively modified due to tyrosine phosphorylation. The membrane-proximal α-helix is slightly longer (K\textsuperscript{716}-K\textsuperscript{738}), however the C-terminal region directly following pY\textsuperscript{747} is no longer helical except for some helical tendencies in A\textsuperscript{790}-T\textsuperscript{793} region. Another surprising finding is that the kink at residues D\textsuperscript{723}/R\textsuperscript{724} in β\textsubscript{3}NP, which allows the helix to bend, bringing the flexible loop (K\textsuperscript{738}, A\textsuperscript{742}) into possible contact with the membrane surface, has shifted toward the residues K\textsuperscript{727}/E\textsuperscript{728} in β\textsubscript{3}MP and β\textsubscript{3}BP. The angles between the two portions of the membrane-proximal helices are not very well defined in all three structures. The recent structural study (21) of the non-phosphorylated β\textsubscript{3} construct, where several additional N-terminal transmembrane residues of β\textsubscript{3} were cross-linked with α\textsubscript{IIb} subunit, has reported that the residue Arg\textsubscript{724} of β\textsubscript{3}CT formed a single-residue hinge and the angle between the two parts of the membrane proximal helix, defined based upon intramolecular NOEs between residues Phe\textsuperscript{277} and Ile\textsuperscript{271}, is about 100°. We however, could not find evidence of the above mentioned NOEs in any of our NOESY experiments. The biological significance of mutual orientation of these two portions of the membrane-proximal helices requires further investigation as it could easily reflect the consequences of higher surface curvature of the micelles, in comparison with mostly flat lipid bilayer.

For β\textsubscript{3}BP in the presence of DPC micelles, the lack of distance restraints (single three-dimensional \textsuperscript{15}N-edited NOESY-HSQC experiment) resulted in inadequate structural convergence. Because of the challenges in preparation of \textsuperscript{13}C,\textsuperscript{15}N-labeled

![Figure 4. Structures of β\textsubscript{3}NP, β\textsubscript{3}MP, and β\textsubscript{3}BP in DPC micelles.](image)

Molecular graphics images were produced by using the UCSF Chimera package (29). A, C, E, backbone superimposition on fifteen lowest energy conformers of β\textsubscript{3}NP, β\textsubscript{3}MP, β\textsubscript{3}BP, respectively; B, D, F, ribbon representation of β\textsubscript{3}NP, β\textsubscript{3}MP, β\textsubscript{3}BP conformers closest to the mean structures. The intensity ratios from the PRE experiments (β\textsubscript{3}NP/MP/BP + 16-DSA + DPC) are mapped onto the surfaces of β\textsubscript{3}NP, β\textsubscript{3}MP, and β\textsubscript{3}BP respectively. Green to orange to white color gradient is used to map the PRE intensities (respective color keys are shown adjacent to the figure). Overlapping residues, residues with missing information, and prolines are marked in gray.
\( \beta_3\text{BP} \) we could not perform 3D \(^{13}\text{C}\) -edited NOESY-HSQC experiment. To circumvent this issue, since the chemical shifts for the N-terminal residues (K\(^{716}\)-A\(^{750}\)) between \( \beta_3\text{MP} \) and \( \beta_3\text{BP} \) were virtually identical (Fig. 1, B and D), we introduced the additional distance restraints corresponding to these N-terminal amino acids from \( \beta_3\text{MP} \) in \( \beta_3\text{BP} \) structure calculations. These additional restraints have resolved the issue of convergence. As expected, the structure is very similar to \( \beta_3\text{MP} \) except a slightly sharper kink in membrane proximal helix and the orientation of C terminus residues (752S-G762). The crucial differences in C terminus arise due to the phosphorylation of Tyr755, which probably affects the orientation of the 756NITRY760 motif. In contrast to \( \beta_3\text{BP} \), where Tyr759 interacts with the membrane (for more details, see below), in the case of \( \beta_3\text{MP} \), pY759 is pointing in opposite direction away from the membrane due to the repulsion between the negatively charged phosphate groups of pY759 and DPC.

To determine how tyrosine(s) phosphorylation alters the membrane binding, we utilized the PRE approach. Two paramagnetic relaxation agents which selectively partition in hydrophobic environment, 5-doxyl stearic acid (5-DSA) and 16-DSA (24) were introduced into the DPC micelles and the consequent drop in the intensities of the amide peaks of all three \( \beta_3 \) constructs was monitored. The doxyl moiety in 16-DSA is attached to the very end of the aliphatic chain and thus gets localized at the center of DPC micelles. In 5-DSA, on the other hand, the doxyl moiety is situated close to the polar head group and the membrane-water interface. Both these tags were utilized to determine the membrane-embedded residues. Supplemental Fig. S6 represents the intensity ratios of the backbon amide groups of \( \beta_3\text{NP}/\text{MP}/\text{BP} \) upon titration with 5-DSA and 16-DSA and Fig. 4, B, D, and F depict these ratios (selected for 16-DSA) mapped on the surfaces of \( \beta_3\text{NP}/\text{MP}/\text{BP} \) structures representing the direct contacts with the micelles. In the case of \( \beta_3\text{NP} \), we confirmed some of our earlier intermolecular NOEs findings (8). The membrane-proximal residues (L717-I721) and Tyr747 of \( \beta_3\text{NP} \) are, indeed, inserted into the membrane. Moreover, the region 738KWD740 is associated with the membrane surface judging by the drop in intensity ratios upon titration with 5-DSA, but not with 16-DSA (supplemental Fig. S6, A and B). Surprisingly, the C-terminal 756NITRY760 motif of \( \beta_3\text{NP} \) also shows significant drop in peaks intensities in both cases (5-DSA and 16-DSA) and is probably associated with the membrane. Previously we could not detect any intermolecular NOEs to support this finding, which is due to the highly dynamic nature of this C-terminal region as confirmed by \(^{15}\text{N}\) relaxation data (supplemental Fig. S7). Although the intensity ratios are rather similar for all \( \beta_3 \) constructs, we do see specific differences in the patterns of membrane association upon tyrosine phosphorylation. In \( \beta_3\text{MP} \), as in the case of \( \beta_3\text{NP} \), the membrane-proximal residues (L717-I721) are membrane embedded and the C-terminal 756NITRY760 motif is either membrane embedded or associated. However, unlike \( \beta_3\text{NP} \), neither 738KWD740 nor pY747 are inserted into or associated with the membrane (supplemental Fig. S6, C, D), which is, most probably, a direct result of tyrosine phosphorylation. The charge repulsion between the negatively charged phosphate groups of \( \beta_3\text{MP} \) and DPC may not allow the pY747 to come within close proximity to the membrane even in the presence of counterbalancing positively charged choline group. This, in turn, might affect the orientation of 738KWD740 motif, resulting in Trp739 pointing in opposite direction from the membrane (Fig. 4, C and F). In \( \beta_3\text{BP} \), similar to \( \beta_3\text{NP}/\text{MP} \), the membrane-proximal residues (L717-I721) are inserted into the membrane. However, due to the phosphorylation of both tyrosines, neither the 738KWD740 nor 756NITRY760 motifs are inserted or associated with the membrane. In summary, we have not only confirmed our earlier findings about \( \beta_3 \) interaction with phospholipids but also have gained some novel insights into how tyrosine phosphorylation affects these interactions and causes major conformation changes.

**DISCUSSION**

Many integrins share highly conserved membrane proximal helical regions (25, 26) containing GFFKR/HDR(R/K)E sequences in the \( \beta \) subunits. Earlier we have shown (7) that under aqueous conditions the membrane-proximal regions of \( \alpha_{\text{IIb}}\beta_3 \) integrin form a heterodimeric complex maintained by a rather famous inter-subunit clasp between residues R\(^{995}\)(\( \alpha_{\text{IIb}} \)) - D\(^{723}\)(\( \beta_3 \)) along with several other hydrophobic (\( \alpha_{\text{IIb}} \) V\(^{996}-\beta_3 \) L\(^{718}\), \( \alpha_{\text{IIb}} \) V\(^{996}-\beta_3 \) I\(^{719}\), \( \alpha_{\text{IIb}} \) F\(^{992}-\beta_3 \) V\(^{721}\)) and electrostatic interactions (salt bridges between 738KWD740 motif, R\(^{995}-\beta_3 \) H\(^{722}\), \( \alpha_{\text{IIb}} \) R\(^{995}-\beta_3 \) E\(^{726}\) (Fig. 3A)). Together these inter-subunit interactions maintain the receptor in the resting state. Recent structures of the transmembrane and cytoplasmic regions of \( \alpha_{\text{IIb}}\beta_3 \) heterodimer under membrane-mimetic conditions confirm this cytoplasmic clasp as an extension of coiled-coil transmembrane domains (27, 28).

Our data show that the phosphorylation of \( \beta_3 \) precludes this weak interaction between \( \alpha \) and \( \beta \) cytoplasmic tails and, therefore, may play a critical role in maintaining the active state of the receptor during outside-in signaling. Closer examination of \( \beta_3\text{MP} \) structure reveals several features supporting this conclusion. The superimposition of \( \beta_3\text{MP} \) over \( \beta_3\text{NP} \) in complex with \( \alpha_{\text{IIb}} \) (PDB ID 1M8O) shows severe steric clashes between \( \beta_3\text{MP} \) and the \( \alpha_{\text{IIb}} \) subunit (Fig. 5A). In addition, the heterodimeric complex of integrin tails involves both hydrophobic and electrostatic interactions with Asp\(^{723}\) and Glu\(^{726}\) of \( \beta_3\) CT forming a salt bridge with Arg\(^{995}\) of \( \alpha_{\text{IIb}} \). However, in the case of \( \beta_3\text{MP} \), Asp\(^{723}\) and Glu\(^{726}\) are pointing in opposite direction. Plus, in \( \alpha_{\text{IIb}}\beta_3 \) complex, Leu\(^{719}\) of \( \beta_3 \) is involved in a hydrophobic interaction with Val\(^{990}\) of \( \alpha_{\text{IIb}} \). In \( \beta_3\text{MP} \), however, the membrane proximal hydrophobic residues interact with Trp\(^{739}\) aromatic side chain. Based on our titration experiments, we can speculate that these intramolecular interactions in \( \beta_3\text{MP} \), although weak, are comparatively stronger than the weaker intermolecular interactions necessary to form \( \alpha_{\text{IIb}}\beta_3 \) complex. Because of several technical difficulties, we do not have structural data for the \( \beta_3\text{BP} \) under aqueous conditions. However, based on the similarity of phosphorylation-dependent chemical shift perturbations in the membrane-proximal region (K\(^{716}-\)D\(^{723}\)), we can propose that the \( \beta_3\text{BP} \) maintains a conformation similar to \( \beta_3\text{MP} \). Moreover, the NMR titration experiments for \( \beta_3\text{BP} \) show a significant reduction in the associated chemical shift perturbations, suggesting that the bi-phosphorylation of \( \beta_3\text{BP} \) also favors disruption of the inter-subunit clasp. Our structural
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analysis emphasizes the role of electrostatic interaction between Lys738 and pY747 in the maintenance of the compact fold of phosphorylated β3CT. When expressed in β3-null endothelial cells, K738E, Y747F, and K738E/Y747F mutants diminished cell adhesion response as compared with WT integrin, thus supporting the notion that the phosphorylation-induced fold supports β3 integrin functional activity.

In this study, we also have accumulated the first direct evidence that tyrosine phosphorylation affects the structure and the association of β3CT with membrane. As visualized in Fig. 5B, charge repulsion pushes the phosphorylated tyrosine(s) away from the membrane, probably exposing alternative motifs to interact with different potential integrin-associated proteins, thus providing an additional level of complexity to the regulatory mechanisms employed in integrin signaling. A model for diversity of such interactions is presented in Fig. 5C based on our results. The resting state of the receptor, with the clasp between α and β subunits located within the cytosol is depicted in Fig. 5C, I. When integrin is activated, for example by talin head domain (Fig. 5C, II), α and β subunits are separated. After talin dissociation the re-clasping can be prevented and the activated state of the receptor maintained by tyrosine phosphorylation of the β subunit within cytosol (Fig. 5C, IV) as well as by membrane association of the membrane-proximal helices of a single or both subunits (Fig. 5C, III). The other functionally significant outcome of tyrosine(s) phosphorylation is the redirection of the β subunit mid-region and/or C terminus away from the phospholipid bilayer, thus allowing different adaptor proteins to bind, and, as exemplified in the case of Shc (Fig. 5C, V), propagating outside-in signaling events within cytoplasm.

To conclude, in this study, we have performed detailed NMR analysis of the effects of tyrosine(s) phosphorylation on integrin β3CT under both aqueous and membrane-mimetic conditions. We have shown that the phosphorylation causes significant conformational rearrangement in β3CT under solution conditions where the pY747 containing segment folds back and interacts with the membrane-proximal region. This arrangement prevents the β3CT from binding to ε11001CT, thus likely dictating an unclasped state of the receptor necessary to mediate integrin outside-in signaling. Moreover, tyrosine(s) phosphorylation under membrane-mimetic conditions modifies β3CT’s interaction with the membrane and perturbs its overall fold. By preventing the phosphorylated tyrosines containing regions from being inserted into or associated with the lipid bilayer phosphorylation might shift the equilibrium of integrin interactions with the different cytoplasmic adaptor proteins, adjacent receptors, and/or cytoskeleton. Our data provide a structural basis for the critical role of Tyr747 phosphorylation in controlling β3CT function and shed light upon molecular details of how phosphorylation may play multiple roles in regulating different states of cell surface receptors, suggesting a more complex paradigm than a simple two state (active/inactive) model.

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