Talin phosphorylation sites mapped by mass spectrometry

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Talin, a 270 kDa protein containing 2541 amino acids, forms a direct link between integrins and the cytoskeleton (Critchley, 2004; Critchley, 2000). Protein domains of talin include an N-terminal globular head and a C-terminal rod. The globular head contains sequences that resemble the FERM domain found in the ERM (exrin, radixin and moesin) family of proteins (Rees et al., 1990). FERM domains contain three subdomains (F1, F2 and F3). The major integrin-binding site of talin lies within the F3 subdomain and involves a PTB (phosphotyrosine-binding) domain that interacts with the NPxY motif present in the cytoplasmic tails of the integrin β subunit (Calderwood et al., 2002; Garcia-Alvarez et al., 2003). The F3 subdomain of talin also binds type I PIP kinase γ (phosphatidylinositol-4*P* 5-kinase) at a site whose sequence overlaps with that found in the cytoplasmic tails of the integrin β subunit (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002; de Pereda et al., 2004). Binding of the integrin cytoplasmic tails to the FERM domain of talin induces conformational changes in the integrin extracellular domains, thus increasing their affinity for ligands (Calderwood et al., 2004; Tadokoro et al., 2003; Calderwood et al., 2002; Kim et al., 2003). Conversely, PIP kinase γ inhibits integrin binding to talin F3 (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002). The talin FERM domain also contains a binding site for focal adhesion kinase (FAK), a non-receptor tyrosine kinase involved in integrin outside-in signaling, and layilin, a hyaluronon-binding membrane protein (Bono et al., 2001; Borowsky and Hynes, 1998). Both the FERM and rod domains of talin possess binding sites for actin (Hemmings et al., 1996; Senetar et al., 2004; McCann and Craig, 1997; Lee et al., 2004). Binding sites on the rod domain of talin include one thought to be involved in homodimerization (Molony et al., 1987; Muguruma et al., 1995), three for interaction with the cytoskeletal protein, vinculin (Bass et al., 2002; Bass et al., 1999; Izard et al., 2004; Papagrigoriou et al., 2004), and one for a second interaction with integrins (Xing et al., 2001; Tremuth et al., 2004).

Phosphorylation of the FERM family of proteins regulates their interactions with transmembrane proteins (Tsukita and Yonemura, 1999). Talin is also phosphorylated (Qwarnstrom et al., 1991; Tidball and Spencer, 1993; Turner et al., 1989; Watters et al., 1996; Beckerle et al., 1989) and the integrin-binding site (Yan et al., 2001; Martel et al., 2001) and at least one vinculin-binding site (Papagrigoriou et al., 2004) in talin are masked. This suggests that conformational rearrangements in talin could be required for integrin activation and interactions with vinculin in a manner analogous to other ERM proteins. Talin is an in vitro substrate of protein kinase C (PKC) and is phosphorylated on both serine and threonine residues in blood platelets (Litchfield and Ball, 1990; Litchfield and Ball, 1986; Murata et al., 1995). These observations point to the possibility that talin phosphorylation could be of importance for regulating its biological functions such as integrin activation. Talin is also cleaved between the head and the rod domains by the enzyme calpain. This enhances the binding of talin to the β3 integrin subunit cytoplasmic tail (Yan et al., 2001). Phosphorylation of talin could be a mechanism for altering the availability of the cleavage site to calpain. In addition, given the number of protein partners that talin interacts with, talin phosphorylation could control when and where these interactions occur. Here we use a combination of immobilized metal affinity chromatography and tandem mass spectrometry to map the phosphorylation sites of talin isolated from human blood platelets.

We chose to isolate talin from platelets because it is highly abundant in this source and easily separated from other platelet proteins by SDS-PAGE. Talin gives rise to a complex mixture of peptides upon digestion with proteolytic enzymes. To cover 95.1% of the talin sequence and 96.4% of the serine, threonine and tyrosine residues, we performed three separate digests with three different combinations of enzymes (Lys C/trypsin, Lys C/Asp-N and Lys C/GluC [for data, see the Cell Migration Gateway (http://www.cellmigration.org/resource/discovery/proteomics/talin_total.shtml)].

We used cell-permeable phosphatase inhibitors, calyculin A and sodium peroxivanadate to suppress de-phosphorylation of the serine/threonine and tyrosine residues, respectively, and, thus, to increase the abundance of the corresponding phosphopeptides. Platelets were pretreated with the phosphatase inhibitors and stimulated with agonist (TRAP) for 30 seconds. Only three sites of phosphorylation, T144, T150 and S446, were detected when the mixture of peptides from each of the three enzymatic digests of talin was analyzed directly by tandem mass spectrometry. Stoichiometries for each these three sites were in the range of 40-60%. The location of these high stoichiometry sites in relation to the domain structure of talin is shown in Fig. 1. TRAP is the N-terminal peptide sequence liberated as a consequence of cleavage of PAR-1, the major human platelet thrombin receptor. It is a potent agonist of platelets, mimicking the action of thrombin. PAR-1 is a G-protein-coupled receptor that activates G_q, G_{12} and perhaps G_i family members, which leads to the activation of PLCβ, PI 3-kinase and the monomeric G proteins Rho, Rac and Rap1, causes an increase in the cytosolic Ca^{2+} concentration and inhibits cAMP formation (Brass, 2003). The phosphorylations reported were an aggregate of those observed in talin from platelets stimulated for 5, 10, 30 and 60 seconds with TRAP.

Subsequently, we employed immobilized metal affinity chromatography (IMAC) to

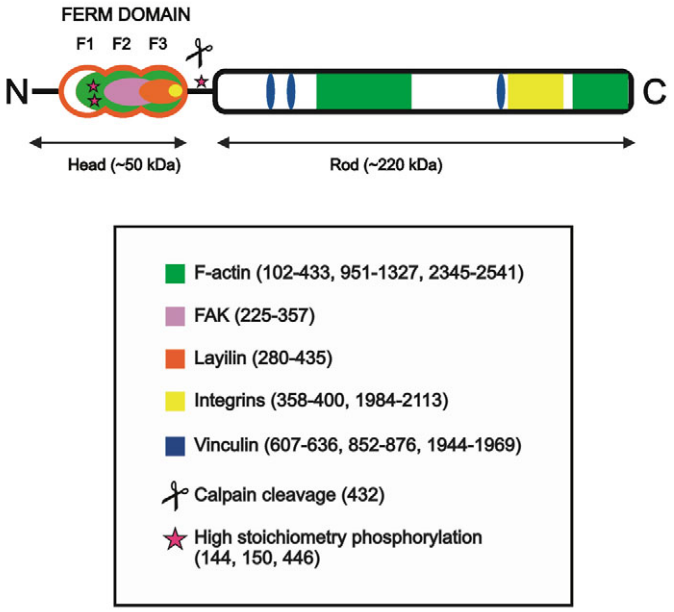

Fig. 1. Domain structure of talin. The three modules comprising the FERM domain (F1, F2, F3), calpain cleavage site and rod domain are depicted. The locations of binding sites for actin, focal adhesion kinase (FAK), layilin, integrin b3 and vinculin are shown. Sites of high stoichiometry phosphorylation (Thr144, Thr150 and Ser446) are indicated by red stars.

Table 1. Summary of human platelet talin phosphorylation sites

Peptide	Residue	Abundance	Predicted kinase
VALpSLK	5	++	PKC, CKI
TMQFEPSTMVpYDACR	26	+	–
ALDpYYMLR	70	+	–
NGDpTMEYR	78	++	–
MLDGpTVK	96	++	PKC
TVTDMLMpTICAR	114	+	–
IGITNHDEpYSLVR	127	++	SRC
IGITNHDEYpSLVR	128	++	CKI
KEEITGpTLR	144	++++	PKC
DKpTLLRDEK	150	++++	PKC
EQGVEEHEpTLLLR	190	+++	–
TYGVpSFFLVK	311	+	–
STMLEDSVpSPK	425	+++	CDK 5
KpS*pT*VLQQQYNR	429 or 430	++	PKA, PKC, CDC2
KSTVLQQQpYNR	436	++	–
VEHGpSVALPAIMR	446	++++	PKA
pS*GApS*GPENFQVGSMPPAQQQITSGQMHR	455 or 458	++	PKA, PKG, PKC
SGASGPENFQVGpSMPPAQQQITSGQMHR	467	++	DNAPK, PKC
AVApSAAAALVLK	677	+	PKC
NIFSpSMGDAGE	815	+	CKII, CKI
QAAASATQTIAAAQHAASpTPK	941	++	PKC
GSQAQPDpS*PpS*AQLALIAASQSFLQPGGK	979 or 981	+	MAPK, CDC2, GSK3
CVpSCLPGQR	1201	++	–
HTSALCNpSCR	1508	+	PKC
TVpSDSIK	1641	++	CKII, PKC, RSK
DLDQASLAAVpSQQLAPR	1684	+	DNAPK
VMVTNVTpSLLK	2127	+	–
EADEpSLNFE	2338	++	CKII, DNAPK
LAQIRQQQpYK	2530	++	–
FLPpSELRDEH	2535	++	CKII

Talin was isolated from platelets that were stimulated with TRAP and treated with phosphatase inhibitors. Phosphopeptides were enriched from enzymatic digests of talin by IMAC and analyzed by tandem mass spectrometry. Relative phosphopeptide abundance is expressed in terms of calculated area for all observed charge states. The most abundant phosphopeptides are displayed as ++++ (phosphorylation stoichiometries estimated to be at the 40-60% level). Those that exhibit peak areas decreased by a factor of 10,100, and 1000 are shown as +++, ++ and +, respectively. Kinases predicted by NetPhos 1.0 (www.cbs.dtu.dk/services/NetPhosK/) and Scansite (scansite.mit.edu) to phosphorylate the identified sites are shown.

Phosphorylated sites are indicated in bold.

*Specific residue phosphorylated unclear.

enrich the above mixtures for phosphopeptides and then repeated the analysis by tandem mass spectrometry. The 30 phosphorylation sites detected by this approach are shown in Table 1. Note that the additional 27 sites have stoichiometries that are 10-1000-fold lower than the sites detected without enrichment by IMAC. We used NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and Scansite (http://scansite.mit.edu/) to predict the kinases that might be responsible for phosphorylation of particular sites (Table 1). The identified sites are potential targets for kinases involved in a variety of physiological processes. For example, T144, T150 and S425 are potentially phosphorylated by protein kinase C (PKC), whereas S446 is predicted to be phosphorylated by PKA, although with a low score by NetPhos (Table 1). S425 can also be potentially phosphorylated by CDK5.

Materials and Methods

Platelets were obtained by centrifugation of fresh platelet concentrate at 850 ***g*** for 20 minutes at room temperature, washed by centrifugation at 850 ***g*** at room temperature and resuspended in Tyrode's Buffer (5 mM HEPES, 137 mM NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 1 mg/ml BSA, 1 mg/ml glucose, pH 7.4). Approximately 10^9 platelets were incubated at 37°C for 30 minutes, following which Calyculin A and peroxovanadate were added to 50 nM and 0.1 mM, respectively, and the resulting mixture was incubated at 37°C for an additional 30 minutes. Platelets were centrifuged at 850 ***g*** for 15 minutes, resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and mixed with TRAP (thrombin receptor agonist peptide, SFLLRN) (Bachem, King of Prussia, PA) to achieve a concentration of 100 μM for 5, 10, 30 and 60 seconds or left untreated. Aliquots of this mixture corresponding to 10^8 platelets were then mixed with an equal volume of the SDS-PAGE sample buffer supplemented with phosphatase inhibitors (50 mM NaF, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate) and immediately incubated at 95°C for 5 minutes. Samples were fractionated on a precast polyacrylamide gels (5% Criterion gels, BioRad, Hercules, CA) and the talin band was visualized by Coomassie staining (Bio-Safe Coomassie, BioRad, Hercules, CA), excised, destained and subjected to in-gel digestion according to a published protocol (Shevchenko et al., 2002; Shevchenko et al., 1996; Wilm et al., 1996), with slight modifications that did not influence the final outcome (C.P. and D.F.H., unpublished). The resulting peptides were enriched for phosphopeptides by immobilized metal affinity chromatography (IMAC) (Ficarro et al., 2002) and then analyzed by high performance liquid chromatography (HPLC) interfaced to electrospray ionization on tandem mass spectrometers (LCQ Deca XP or LTQ-FT, Thermo Electron). To obtain a rough approximation of stoichiometry of phosphorylation, ion currents for the IMAC-bound phosphopeptide and the corresponding non-phosphorylated peptide in the IMAC flow through were measured and used to estimate the percentage of peptide phosphorylated.

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