Cortactin phosphorylation sites mapped by mass spectrometry

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Cortactin is a scaffolding protein that is targeted to sites of actin polymerization, including lamellipodia in migrating cells, cell-cell junctions in epithelial cells, growth cones of neurons, podosomes of osteoclasts, invadopodia of tumor cells, and sites of actin rearrangement induced by pathogenic bacteria and viruses (Daly, 2004; Lua and Low, 2005; Pfaff and Jurdic, 2001). Although it is phosphorylated in response to a number of stimuli including growth factors, bacterial invasion, platelet activation, transformation and integrin-mediated adhesion, the only sites of phosphorylation that have been characterized to date are Y421, Y466 and Y482 (Src sites that regulate cell migration) and S405 and S418 (ERK sites that regulate N-WASP-mediated actin polymerization) (Campbell et al., 1999; Huang et al., 1998; Martinez-Quiles et al., 2004; Weed and Parsons, 2001). To better understand how the phosphorylation of cortactin contributes to the dynamic regulation of actin structures, we mapped the repertoire of phosphorylation sites within cortactin by mass spectrometry (MS). Herein, we report the identification of 17 new sites of phosphorylation (12 serine, four threonine and one tyrosine residues). The distribution of phosphorylation sites on well-conserved residues within cortactin supports a role for these modifications in the regulation of cortactin function.

Insights into the role of phosphorylation come from the analysis of the domain structure of cortactin which includes an N-terminal acidic (NTA) domain, a repeat domain (comprised of 6.5 copies of a 37 amino acid sequence), an α-helical and proline-rich (HPR) domain and a C-terminal Src homology 3 (SH3) domain (Fig. 1). The NTA domain of cortactin nucleates actin polymerization through direct interaction with the Arp2/3 complex (Weed et al., 2000). The NTA domain shares homology with the acidic domains found in other Arp2/3-binding proteins, and mutation of the conserved tryptophan (W22A) or the two adjacent aspartic acids (D20G and D21A) within the NTA of cortactin disrupts the interaction with the Arp2/3 complex and inhibits the nucleation of new actin filaments (Uruno et al., 2001; Weaver et al., 2003). The MS analysis showed that the conserved DDW sequence was flanked by three sites of phosphorylation (S11, T13 and T24) (Fig. 2A). Peptides containing phosphorylated S11 or T13 were among the most abundant peptides identified in this analysis (Table 1). Residues S11 and T24 are conserved between mouse, human, frog and fish cortactin sequences; residue T24 is maintained in fly and sponge (Table 2). The proximity of these phosphorylation sites to the DDW motif indicates that phosphorylation of S11, T14 and/or T24 contributes to the regulation of the association of cortactin with the Arp2/3 complex.

The repeat domain binds directly to filamentous (F)-actin, providing a branch point for nascent actin polymerization (Wu and Parsons, 1993). This domain contained seven sites of phosphorylation (S135, S150, S172, T182, S209, S282 and S283). Fig. 2B shows the alignment of the individual Repeats, with the phosphorylated sites shown in red. The same conserved serine (SAVG) was phosphorylated in four of the six repeats. Since the repeat domain is both necessary and sufficient for F-actin binding, we suggest that modification of sequences within this domain influences the structure of each repeat and plays a role in regulating the binding of cortactin to actin filaments.

Within the HPR domain, we detected seven sites of phosphorylation (S322, S345, S348, S407, S454, T468 and Y475) (Table 2). The peptide that was phosphorylated on S322 was one of the most abundant peptides identified. An additional site was mapped in peptides containing S417, S418 and Y421, but the data were ambiguous with respect to which residue(s) was phosphorylated. Previous studies have shown that cortactin is phosphorylated on S418 and Y421 (Campbell et al., 1999; Huang et al., 1998). It is possible that phosphorylation of these residues in the HPR contributes to conformational regulation of the SH3 domain or accessibility to the SH3 domain. The SH3 domain binds a number of effector molecules, including proteins that regulate actin polymerization (WIP, N-WASP,), endocytosis and vesicle trafficking (dynamin-2), GTPase regulators (FGD1, BPGAP1) and adaptor and/or scaffold proteins (CD2AP, CortBP1, ZO-1 and Shanks) (Daly, 2004; Lua and Low, 2005; Weed and Parsons, 2001). No sites of phosphorylation were identified in the C-terminal SH3 domain.

Fig. 1. Distribution of phosphorylation sites in cortactin. The domain structure of cortactin is shown with the mapped sites of serine (blue stars), threonine (green stars), and tyrosine (red stars) phosphorylation shown below.

Table 1. Relative abundance of modified peptides identified by mass spectrometry

Residue	Cortactin peptide sequence (mouse)	Relative abundance (+) Inhibitors	Relative abundance (–) Inhibitors
S11	ASAGHAVSITQDDG	IMAC	–
S11	KASAGHAVSITQDDGGADDW	++	–
S11	KASAGHAVSITQDDGGADDWETDPDFVNDVSEKEQRW	++++	–
S11	ASAGHAVSITQDDGGADDWETDPDFVNDVSEK	++++	–
[S11-T13]	KASAGHAV[SIT]QDDGGADDWETDPDFVNDVSEKEQRW	+++	–
T13	ASAGHAVSITQDDG	IMAC	–
T13	ASAGHAVSITQDDGGADDWETDPDFVNDVSEK	++++	–
T13	ASAGHAVSITQDDGGADDWETDPDFVNDVSEKEQRWGAK	++++	–
T24	ASAGHAVSITQDDGGADDWETDPDFVNDVSEK	IMAC	–
S135	GVQMDRVDQSAVGF	IMAC	–
S150	EYQGKTEKHASQKDY	IMAC	–
S172	GVQADRVDKSAVGF	++	IMAC
T182	DYQGKTEKHESQKDY	IMAC	–
S209	GIDKDKVDKSAVGF	IMAC	IMAC
S282	GVQSERQDSSAVGF	IMAC	–
S283	GVQSERQDSSAVGF	IMAC	++++
S283	FGVQSERQDSSAVGF	IMAC	–
S322	GVQKDRMDKNASTFEEVVQVPSAY	++++	–
[S322-T323]	NA[ST]FEEVVQVPSAYQK	+	–
S345	QKTVPIEAVTSKTSNIRANF	IMAC	–
S348	QKTVPIEAVTSKTSNIRANF	IMAC	IMAC
S407/[S417-Y421]	KQTPPASPSPQPIEDRPP[SSPIY]EDAAPF	++	–
[S417-S418]	RPP[SS]PIYEDAAPF	IMAC	–
[S417-S418]	DRPP[SS]PIYEDAAPF	IMAC	–
S454	KAEPSYRGSEPEPEYSIEAAGIPEAGSQQGLTY	++	–
T468	ETTEAPGHYQAEDDTY	IMAC	–
Y475	ETTEAPGHYQAEDDTY	IMAC	–

Each of the modified peptides identified from cortactin is listed, with the phosphorylated residue(s) shown in red. Phosphorylation sites in brackets could not be specified unambiguously. In most experiments, the cells were treated with phosphatase inhibitors before lysis. Relative peptide abundance is expressed in terms of peak areas observed for all charge states of particular peptides. Peak areas for the most abundant phosphopeptides are displayed as ++++; those with areas that are decreased by a factor of 10, 100 and 1000 are shown as +++, ++ and +, respectively. Phosphopeptides that were not identified without inhibitor treatment are indicated as –. Peptides labeled 'IMAC' were only observed after enrichment of the sample by immobilized metal-affinity chromatography.

A

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mwKASAGHAV SITQDDGGAD DWETDPDFVN DVSEKEQRWG AKTVQGSGHQ  50
EHINIHKLRE NVFQEHQTLK EKELETGPKA SHGYGGKFGV EQDRMDRSAV 100
GHEYQSKlsk HCSQVDSVRG FGGKFGVQMD RVDQSAVGFE YQGKTEKHAS 150
QKDYSSGFGG KYGVQADRVD KSAVGFDYQG KTEKHESQKD YSKGFGGKYG 200
IDKDKVDKSA VGFEYQGKTE KHESQKDYVK GFGGKFGVQT DRQDKCALGW 250
DHQEKLQLHE SQKDYKTGFG GKFGVQSERQ DSSAVGFDYK ERLAKHEPQQ 300
DYAKGFGGKY GVQKDRMDKN A[ST]FEEVVQV PSAYQKTVPI EAVTSKTSNI 350
RANFENLAKe reqedrrkae aeraqrmakE RQEQEEARRK LEEQARAKKQ 400
TPPASPSPQP IEDRPP[SSPI Y]EDAAPFKAE PSYRGSEPEP EYSIEAAGIP 450
EAGSQQGLTY TSEPVYETTE APGHYQAEDD TYDGYESDLG ITAIALYdyq 500
aagddeisFD PDDIITNIEM IDDGWwRGVC KGRYGLFPAN YVELRQ     546
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B

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Repeat 1: ASHGYGGKFGVEQDRMDRSAVGHEYQSKLSKHCSQVD
Repeat 2: SVRGFGGKFGVQMDRVDQSAVGFEYQGKTEKHASQKD
Repeat 3: YSSGFGGKYGVQADRVDKSAVGFDYQGKTEKHESQKD
Repeat 4: YSKGFGGKYGIDKDKVDKSAVGFEYQGKTEKHESQKD
Repeat 5: YVKGFGGKFGVQTDRQDKCALGWDHQEKLQLHESQKD
Repeat 6: YKTGFGGKFGVQSERQDSSAVGFDYKERLAKHEPQQD
Repeat 7: YAKGFGGKYGVQKDRMDK
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The MS analysis described herein adds to the repertoire of post-translational modifications that occur in cortactin. Previous studies had mapped sites of phosphorylation to S405, S418, Y421, Y466 and Y482 (Campbell et al., 1999; Huang et al., 1998). Of these, S418 and Y421 were identified as potential sites in the current study; the other sites were not seen under the conditions of these experiments. However, 17 new sites of phosphorylation were identified that were found in all domains of cortactin except the SH3 domain. Including the previously identified sites, 22 sites of phosphorylation in cortactin are currently known. The task

Fig. 2. Identified sites of phosphorylation in cortactin. (A) The sequence of mouse cortactin (GenBank accession no. BC011434) is shown with the amino acids that were identified by mass spectrometry (MS) shown in capital letters. Peptides representing 93% of the cortactin protein were identified. Phosphorylated residues identified by the MS analysis are shown in red. Bracketed residues contain a phosphorylation site that could not be specified unambiguously. (B) The 37 amino acid Repeats are aligned showing the sites of phosphorylation (red).

Table 2. Cortactin phosphorylation sites shared between species

Residue		Human	Frog	Fish	Fly	Sponge
NTA	S11	+	+	+	–	–
	T13	–	–	–	–	–
	T24	+	+	+	+	+
Repeat	S135	+	+	+	+	+
	S150	+	+	+	+	+
	S172	+	+	–	+	–
	T182	+	+	+	–	–
	S209	+	+	+	–	–
	S282	+	–	+	–	+
	S283	–	–	+	–	+
HPR	S322	+	–	+	–	–
	[T323]	+	–	+	–	–
	S345	+	+	+	–	–
	S348	+	+	+	–	–
	S407	–	–	–	–	–
	[S417]	+	–	–	–	–
	[S418]	+	+	–	–	–
	[Y421]	+	+	–	–	–
	S454	+	–	–	–	–
	T468	–	–	–	–	–
	Y475	+	+	–	–	–

The sequence of mouse cortactin was compared to the cortactin sequences from human, frog (*Xenopus laevis*), fish (*Danio rerio*), fly (*Drosophila melanogaster*) and sponge (*Suberites domuncula*) (van Rossum et al., 2005). +, phosphorylated amino acids that are conserved; –, phosphorylated amino acids that are not conserved. Phosphorylation sites on residues in brackets could not be specified unambiguously.

is now to determine the differential regulation and functional consequences of these modifications in cortactin.

Materials and Methods

HEK293 cells were transfected with FLAG-cortactin plasmid (5 μg/ 10^7 cells) using Polyfect (QIAGEN). The cells were treated for 30 minutes with the serine/threonine phosphatase inhibitor calyculin (0.1 μM), the tyrosine phosphatase inhibitor sodium ortho-vanadate (5 mM) or were processed without any inhibitor treatment. FLAG-cortactin was captured from clarified cell extracts made in CSK-NP buffer [150 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 10% glycerol, 10 mM sodium ortho-vanadate, 2 mM sodium pyrophosphate, 1% NP-40, 1× EDTA-free protease inhibitors (Roche)] using M2-agarose affinity resin (anti-FLAG antibody conjugated to agarose, Sigma). After 2 hours of incubation at 4°C, the M2 agarose beads were sequentially washed with CSK-NP buffer, medium-salt buffer (150 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4) and high-salt buffer (1 M NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4) to remove non-specific binding proteins. The cortactin protein was eluted with 0.3 mg/ml of FLAG peptide in MS buffer. Typically 30-40 pmoles of purified cortactin were recovered from 10^7 cells, and 2-10 pmoles of protein were used to initiate C18 reverse-phase (RP)-HPLC analysis and mass spectrometry. Aliquots of purified cortactin protein were digested with either chymotrypsin or Lys-C followed by chymotrypsin to maximize the coverage of peptides analyzed in the mass spectrometer. The resulting peptides were separated by RP-HPLC coupled to online detection by nanoflow LC, microcapillary ESI tandem mass spectrometry (LTQ-FTMS, Thermo Electron, San Jose, CA). In some cases enrichment of phosphopeptides was performed with immobilized metal affinity chromatography (IMAC) (Ficarro et al., 2002), prior to RP chromatography. In those cases, 10-30 pmoles of protein were loaded onto IMAC columns.

Individual MS/MS spectra from each analysis were searched against a FASTA database containing the protein sequence for mouse cortactin using the SEQUEST database-searching algorithm. The SEQUEST parameters file included a differential search for +80 atomic mass unit (amu) on serine, threonine and tyrosine residues to identify sites of phosphorylation. Oxidized methionine was identified by a differential search of +16 amu on the methionine residue. Static modification of +57 amu on cysteine was due to treatment with the protein with DTT-iodoacetamide before the proteolytic digest. For IMAC analyses, static modifications of +14 amu on glutamate, aspartate and the C-terminus of the peptide were included to account for conversion of carboxylic acid groups to methyl esters. Peptides that scored above Xcorr 3.0 and met the criteria described by the Yates group (Washburn et al., 2001), were deemed as valid hits. All hits that included a phosphorylation site were verified by manual interpretation of the corresponding MS-MS spectra.

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