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Identification of Phosphorylation Sites within the Signaling Adaptor APPL1 by Mass Spectrometry

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Abstract

APPL1 is a membrane-associated adaptor protein implicated in various cellular processes, including apoptosis, proliferation, and survival. Although there is increasing interest in the biological roles as well as the protein and membrane interactions of APPL1, a comprehensive phosphorylation profile has not been generated. In this study, we use mass spectrometry (MS) to identify 13 phosphorylated residues within APPL1. By using multiple proteases (trypsin, chymotrypsin, and Glu C) and replicate experiments of linear ion trap (LTQ) MS and LTQ-Orbitrap-MS, a combined sequence coverage of 99.6% is achieved. Four of the identified sites are located in important functional domains, suggesting a potential role in regulating APPL1. One of these sites is within the BAR domain, two cluster near the edge of the PH domain, and one is located within the PTB domain. These phosphorylation sites may control APPL1 function by regulating the ability of APPL1 domains to interact with other proteins and membranes.

Keywords

Phosphoproteomics; Linear Ion Trap Mass Spectrometry; Orbitrap mass spectrometry; Phosphorylation; Phosphorylation site mapping; APPL1

Introduction

Adaptor protein containing a PH domain, PTB domain and Leucine zipper motif (APPL1) is a 709 amino acid membrane associated protein that has been reported to play a key role in the regulation of apoptosis, cell proliferation, cell survival, and vesicular trafficking.^{1, 2} APPL1 is widely expressed and found in high levels in the heart, brain, ovary, pancreas, and skeletal muscle.¹ Although a significant amount of interest has been generated in the interactions and function of APPL1, the complete phosphorylation profile of this protein has not been described.

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Supporting Information Table 1 of putative kinases that phosphorylate APPL1 and all raw MS/MS spectra can be found in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

To date, phosphorylation of three residues, threonine 399, and serines 401 and 691, which were identified from global profiling studies³⁻⁷ are reported in protein databases, including Phosphosite, Proteinpedia/Human Protein Reference Database, and Expasy-SwissProt.

APPL1 mediates its function through a series of domains, including an N-terminal Bin-Amphiphysin-Rvs (BAR), a central Pleckstrin homology (PH), and a C-terminal phosphotyrosine binding domain (PTB).^{1, 8} Both the BAR and PH domains are involved in binding to cell membranes. The BAR domain is a dimerization motif associated with the sensing and/or induction of membrane curvature while the PH domain binds to phosphoinositol lipids.^{9, 10} The BAR domain has also been shown to be critical in the ability of APPL1 to localize to endosomal structures.¹¹ In APPL1, the BAR and PH domains are thought to act together as a functional unit forming an integrated, crescent-shaped, symmetrical dimer that mediates membrane interactions.^{12, 13} Moreover, the BAR and PH domains function together to create the binding sites for Rab5, which is a small GTPase involved in endosomal trafficking.^{13, 14} The C-terminal PTB domain of APPL1 has been shown to be critical in the ability of APPL1 to bind to several signaling molecules, including the serine/threonine kinase Akt, the neurotrophin receptor TrkA, the adiponectin receptors AdipoR1 and AdipoR2, Human Follicle-Stimulating Hormone (FSHR), and the tumor suppressor DCC (deleted in colorectal cancer).^{1, 15-18}

In this study, phosphorylation sites were identified on APPL1 using contemporary MS-based methods, namely by liquid chromatography (LC)-coupled to data-dependent tandem MS on both an LTQMS and LTQ-Orbitrap-MS. The bioinformatic algorithm SEQUEST was used to process the MS/MS data obtained in these phosphorylation mapping experiments. However, spectral assignments required manual validation of all identified phosphorylation site spectra. To obtain near-complete coverage of APPL1, multiple proteases were used in parallel phosphorylation site mapping experiments. Proteolytic digestion with Glu C, trypsin, and chymotrypsin yielded sequence coverages of 44.6%, 88.3%, 81.1%, respectively, with a combined sequence coverage of APPL1 of greater than 99%. A total of 13 phosphorylation sites were detected and four of these sites were found within APPL1 interacting domains, suggesting a potential regulatory role in APPL1 function.

Experimental Procedures

Reagents and Plasmids

FLAG M2-agarose affinity gel, FLAG peptide (DYKDDDDK), and mouse IgG agarose were purchased from Sigma (St. Louis, MO). Calyculin A was purchased from Calbiochem (San Diego, CA). Sodium vanadate was obtained from Fischer Scientific (Fairlawn, NJ). Peroxovanadate was prepared as previously described.¹⁹ FLAG-GFP plasmid was prepared by inserting the FLAG epitope sequence into pcDNA3 (Invitrogen, Carlsbad, CA) and cloning EGFP C1 (Clontech) into the vector at *KpnI* and *BamHI* sites. Human APPL1 (accession number GI:124494248) was then cloned into the FLAG-GFP plasmid at *EcoRI* and the insertion as well as orientation of APPL1 was confirmed by sequencing.

Protein Expression and Proteolytic Digestion

Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin/streptomycin (Invitrogen). HEK-293 cells were transfected with FLAG-GFP-APPL1 (12 µg per 150 mm dish) using Lipofectamine 2000 (Invitrogen). After 36 h, cells were incubated with 1 mM peroxovanadate and 50 nM calyculin A in DMEM with 10% FBS for 30 minutes and extracted with 25 mM Tris, 100 mM NaCl, 0.1% NP-40 (pH 7.4). The lysates were precleared twice with mouse IgG-agarose for 1 h at 4°C, and

immunoprecipitated with FLAG-agarose (Sigma, St. Louis, MO) for 2 h at 4°C. Samples were washed three times with 25 mM Tris, 100 mM NaCl, pH 7.4 and FLAG tagged APPL1 was eluted by incubation of the beads with 0.2 mg/ml FLAG peptide in 25 mM Tris, pH 7.4 for 1 h at 4°C. Purified APPL1 protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The concentration of APPL1 was quantified with a LI-COR Biosciences ODYSSEY Infrared Imaging System using bovine serum albumin (BSA) as a standard.

For MS analyses, APPL1 was separated into three equal aliquots and proteolytically digested by trypsin, chymotrypsin, and Glu C proteases, respectively. Briefly, proteolysis was performed by taking 2.6 µg of APPL1 (20 µl) and diluting to 25 µl with 25 mM ammonium bicarbonate. Cysteine sulfhydryl groups were reduced by the addition of 1.5 µl of 45 mM dithiothreitol (DTT) for 30 min at 55°C followed by alkylation with 2.5 µl of 100 mM iodoacetamide for 30 min at room temperature in the dark. Digestion was performed using 100 ng (1:40 enzyme: substrate, wt:wt) of trypsin gold (Promega, Madison, WI), chymotrypsin (Princeton Separations, Freehold, NJ), or endoproteinase Glu C (Calbiochem EMD Biosciences, Gibbstown, NJ) at 37°C for 16, 4, or 6 h respectively. Proteolysis was quenched by adding 1 µl of 88% formic acid. Subsequently, the digest was lyophilized and then reconstituted in 25 µl of 0.1% formic acid.

Western Blot Analysis

Purified APPL1 protein was subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was incubated with primary antibody against GFP (Invitrogen) or 4G10 (a kind gift from Steve Hanks, Vanderbilt University) at a dilution of 1 µg/ml. The membrane was then incubated with IRDye 800 Conjugated Affinity Purified anti-Rabbit IgG or anti-Mouse IgG (Rockland) at a dilution of 0.1 µg/ml, and visualized using a LI-COR Biosciences ODYSSEY Infrared Imaging System.

Linear Ion Trap and LTQ-Orbitrap MS

LC-MS/MS analyses of APPL1 digests were performed using a linear ion trap mass spectrometer (LTQ, Thermo Electron, San Jose, CA) equipped with an autosampler (MicroAS, Thermo) and an HPLC pump (Surveyor, Thermo), and Xcalibur 2.0 SR2 instrument control. Ionization was performed by using nanospray in the positive ion mode. Spectra were obtained by using data-dependent scanning tandem mass spectrometry in which one full MS scan, using a mass range of 400–2000 amu, was followed by up to 5 MS/MS scans of the most intense peaks at each time point in the HPLC separation. Incorporated into the method was data-dependent scanning for the neutral loss of phosphoric acid or phosphate ($-98\ m/z$, $-80\ m/z$), for which MS³ was performed. Dynamic exclusion was enabled to minimize redundant spectral acquisitions. High resolution data was collected using a similar strategy on a LTQ-Orbitrap mass spectrometer with the exception that the full MS scan was performed in the Orbitrap at 30,000 resolution, rather than at unit mass resolution on the LTQMS. Further instrumental details are available in the Electronic Supplementary Material.

Bioinformatic Analysis

Tandem MS/MS spectra acquired in LTQMS and LTQ-Orbitrap-MS experiments were identified using SEQUEST (University of Washington). MS/MS spectra were extracted from the raw data files into .dta format with spectra containing fewer than 25 peaks being excluded. Files labeled as singly charged were created if 90% of the total ion current occurred below the precursor ion, and all other spectra were processed as both doubly- and triply-charged ions. Proteins were identified using the TurboSEQUEST version 27 (rev. 12) algorithm (Thermo Electron) and the IPI Human database version 3.33 (67837) sequences. Search parameters are outlined in the Electronic Supporting Information. Manual verification was performed on all

phosphorylation assignments having an Xcorr value above 1, 2, and 2.5 for charges +1, +2, and +3, respectively. Validation was performed as previously described.²⁰ All spectra are included in the Electronic Supporting Information according to MIAPE standards.²¹

Results and Discussion

Comprehensive Phosphorylation Map of Human APPL1

In this study, a comprehensive phosphorylation profile of APPL1 is described for the first time. To accomplish this, FLAG-GFP-APPL1 was expressed in HEK-293 cells and subsequently immunoprecipitated for MS analysis according to the purification scheme outlined in Figure 1A. A major band corresponding to the molecular mass of APPL1 was observed when the immunoprecipitate was subjected to SDS-PAGE and stained with Coomassie blue (Figure 1B). The band was confirmed to be APPL1 by Western blot analysis (Figure 1C). Before subjecting APPL1 to MS analysis, we examined the phosphorylation state of this protein using 4G10 phospho-tyrosine antibody. APPL1 was phosphorylated on tyrosine residues as determined by Western blot analysis with 4G10 (Figure 1C). Several other minor bands were detected in the immunoprecipitated samples, which could correspond to endogenous APPL1 or APPL1 binding proteins. However, insufficient peptide signal from MS analyses precluded positive protein identification of these additional minor bands.

At least 13 (as discussed below) phosphorylation sites with 99.6% total amino acid sequence coverage were identified using multiple proteases, including trypsin, chymotrypsin, and Glu C, followed by LC-MS analyses using both an LTQMS instrument and an LTQ-Orbitrap instrument. Of these reported phosphorylation sites, three could not be located to a single amino acid (*i.e.* phosphorylation was determined to exist within a range of potential sites within a peptide).

Table 1 shows each confirmed phosphorylation site assignment by sequence position using the LTQMS instrument. In total, ten phosphorylation sites were identified by combining the data obtained for trypsin, chymotrypsin, and Glu C digests to obtain a sequence coverage of 95.3%. Of these ten sites, only two could not be located to a specific residue, *i.e.* phosphorylation was confirmed to exist between amino acids 97-98 (SS) and 401-403 (SPS). Table 2 shows the confirmed phosphorylation sites using the LTQ-Orbitrap instrument. By combining the data obtained for Glu C, trypsin, and chymotrypsin digests, nine phosphorylation sites were identified with a sequence coverage of 99.6%. Several of these phosphorylation sites were detected in multiple peptides derived from proteolytic miscleavages corresponding to the same site of phosphorylation. Of these nine sites, two could not be located to a specific residue, but were confirmed to exist between amino acids 401-403 (SPS) and 689-691 (SSS). A comparison of the phosphorylation sites identified using the LTQMS and LTQ-Orbitrap yielded four unique sites by the former and three unique sites by the later. We detected five phosphorylation sites, including serines 401/403, 459, 691, 693, and 696 by both methods. Interestingly, most of the phosphorylation sites we detected in human APPL1 are conserved in rat and mouse APPL1 (Table 3), raising the possibility that these sites serve a functional role.

Two of the previously identified phosphorylation sites in APPL1, 401S and 691S, were detected in our analysis while one additional site, 399T, was not definitively assigned. Phosphorylation of 401S was initially identified in epithelial carcinoma (HeLa) cells as part of a large-scale characterization of nuclear phosphoproteins and in an analysis of protein phosphorylation in developing mice brains.^{5, 6} This site was subsequently shown to be phosphorylated in HeLa cells in two additional studies.^{3, 4} Phosphorylation of 691S was detected in HEK-293 cells in response to DNA damage using ionizing radiation.⁷ We also identified phosphorylation of this site in HEK 293 cells under physiological conditions. Phosphorylation at 399T was identified in a global profiling study³, but a positive identification

could not be definitively made in our experiments. Our spectra potentially suggested phosphorylation at 399T, but in these spectra, this site was not the highest confidence assignment. Furthermore, the previous study examined protein phosphorylation during mitosis using HeLa cells arrested in the mitotic phase of the cell cycle while our analysis was performed in HEK-293 cells under conditions in which they were progressing through the cell cycle. Thus, it is possible that phosphorylation of this site is transient if it is regulated by cell cycle progression and difficult to detect.

Phosphorylation Sites within APPL1 Functional Domains

The confirmed phosphorylation sites obtained on both instruments are shown in Figure 2. Of the confirmed sites, four were found in APPL1 interacting domains. Namely serines 97/98 were located in the BAR domain, raising the possibility that phosphorylation at these sites could disrupt APPL1 dimerization as well as endosomal localization. Interestingly, as shown in the crystal structure of the BAR and PH domains, serines 97/98 are located on the concave surface of the BAR domain, which is thought to interact with membranes (Figure 3).^{12, 22} Therefore, phosphorylation at this site could potentially regulate membrane interactions. Serine 374 and tyrosine 378 are clustered near the edge of the PH domain (Figure 3), suggesting a potential link to APPL1 localization. Collectively, these sites in the BAR and PH domains, may contribute to altered APPL1 binding to Rab5, since together these domains are important for this interaction. Finally, tyrosine 604 was found in the PTB domain, which is typically involved in protein-protein interactions, and phosphorylation in this domain may regulate the ability of APPL1 to bind to its interacting protein partners. Interestingly, a significant number of identified phosphorylation sites are found outside of known domains. Even though these sites are outside described domains, it does not imply a lack of functional significance. These sites may have importance in regulating the structure and molecular interactions of APPL1.

Putative Kinases for Identified APPL1 Phosphorylation Sites

Table 4 shows putative kinases for eight of the identified APPL1 phosphorylation sites obtained using NetPhosK 1.0 and Scansite (medium stringency).^{23, 24} These potential kinases include the serine/threonine kinases casein kinase 1 and casein kinase 2, which are involved in Wnt signaling.^{25, 26} Casein kinase 1 is predicted to phosphorylate serines 430, 693 and 696, while casein kinase 2 is predicted to phosphorylate serines 491, 691, and 696. The serine/threonine kinase cell division cycle 2 (cdc2) is an important regulator of cell cycle progression, and is predicted to phosphorylate serines 401, 689, and 691.²⁷ The serine/threonine kinase Akt, also known as protein kinase B, binds to APPL1 via the PTB domain, and is predicted to phosphorylate serine 427 in the area between the PH and PTB domain.¹ Moreover, the serine/threonine kinase glycogen synthase kinase 3 (GSK3), a downstream effector of Akt, is predicted to phosphorylate APPL1 at serine 401.²⁸ Putative kinases obtained from Scansite (low, medium, and high stringency) are shown in Supplementary Table 1.

Advantages and Challenges to Contemporary Phosphoproteomic Methodologies

Figures 4 and 5 demonstrate the necessity of manual verification of bioinformatics analyses. For example, the peptide in Figure 4, GQFVVLSSSQpSEESDLGEGGKKRE, was identified correctly, but because the incorrect peak was used as the monoisotopic peak, the mass error of the precursor ion (-381 ppm) was outside of the acceptable range (-5 to 5 ppm). Conversely, an example of an erroneous SEQUEST assignment is shown in Figure 5. Although b and y ion coverage bracketing the phosphorylation site is sufficient for a high X-corr value and high sequence coverage confidence, high abundance peaks do not correspond to b and y ions or their respective neutral losses. Collectively, these examples and ambiguity arising from gas-phase rearrangement illustrate the continuing need to validate sequencing data in phosphorylation site mapping experiments.^{29, 30}

Conclusion

Emerging data indicate an important role for APPL1 in regulating various cellular processes, such as cell proliferation, apoptosis, and survival, which points to a need to gain insight in to the regulation of this protein.^{1, 2} Since phosphorylation is an important regulatory mechanism, we generated a comprehensive map of phosphorylation sites within APPL1. We detected 13 phosphorylation sites within APPL1, with four of these being identified in functional domains. These sites have potential implications in regulating APPL1 function and interactions, which represents an important avenue for future study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Schematic showing the generalized protocol used for purifying FLAG-tagged proteins. (B) SDS-PAGE gel of immunoprecipitated FLAG-GFP-APPL1 stained with Coomassie blue. Arrow points to purified FLAG-GFP-APPL1. (C) Western blot with GFP-specific antibody (left panel) or phospho-tyrosine antibody (right panel). Left panel shows the purified protein is FLAG-GFP-APPL1 (IB: GFP) and right panel shows that APPL1 is phosphorylated on tyrosine residues (IB: 4G10).

Figure 2.

(A) Phosphorylation sites identified in APPL1, using LTQMS and LTQ-Orbitrap MS. Underlined sites indicate that one phosphorylation is known to exist within the region. (B) A schematic of APPL1 is shown with identified phosphorylation sites relative to the position of APPL1 domains. Interacting regions within APPL1 for several proteins and receptors are also indicated.

Figure 3.

Crystal structure of the BAR and PH domains of APPL1 is shown. Phosphorylation site serine 97/98 and serine 374 are highlighted in red and their positions are indicated with arrows. Note that although tyrosine 378 neighbors the PH domain, it was not included in the crystal structure from the Protein Data Bank (2ELB).

Figure 4.

Tandem MS/MS spectrum acquired using an LTQ-Orbitrap illustrates peak validation for accurate SEQUEST assignments. Inset illustrates a situation whereby the instrument selected the peak at 878.7426 as the monoisotopic peak resulting in erroneous mass accuracy (-381 ppm). Manual validation of the data correctly assigns the accurate monoisotopic peak at 878.4084 resulting in a mass accuracy for the parent species of 0.56 ppm.

Figure 5.

Tandem MS/MS spectrum of a phosphorylation site incorrectly assigned by SEQUEST having the highest Xcorr value of 2.19. SEQUEST assignments report 15% b-ion sequence coverage and 55% y-ion coverage from the y_5 ion to the y_{16} ion. Of the eight most abundant peaks in the spectrum, six ions, indicated by an asterisk, correspond to neither b nor y ions, or to characteristic neutral losses. Manual verification was performed to detect such errors in the bioinformatic assignments. Additionally, the b and y ion coverage fails to bracket the suggested sites of phosphorylation, namely tyrosine 378 and serine 380.

Table 1

Phosphorylation Sites within APPL1 Identified by LTQMS

Peptides ^a	Sequence Position ^b	Protease ^c	[M+H] ⁺ (m/z)
⁹² VIDEL <u>SS</u> CHAVLSTQLADAMMFITQFK ¹¹⁹	‡	Trp	3175.53
³⁷⁶ QI <u>p</u> YLSNPETAAR ³⁸⁹	378Y	Trp	1700.75
³⁹⁰ VNQSALEAVTP <u>SPS</u> FQQR ⁴⁰⁷	‡	Trp	2038.99
⁴⁵⁶ DI <u>p</u> SPVC*EDQPGQAKAF ⁴⁷²	459S	Chymo	1954.93
⁴⁷⁹ TNPFGESEGGSTK <u>p</u> SETEDSILHQLFIVR ⁵⁰⁵	491S	Trp	3029.46
⁵⁹⁵ SESNLSSVC <u>p</u> YIFESNNEGEK ⁶¹⁴	604Y	Trp	2315.94
⁶⁶⁹ LIAASSRPNQASSEGQFVVLP <u>SSS</u> QSEESDLGEGGK ⁷⁰³	689S	Trp	3631.71
⁶⁸³ GQFVVLSS <u>p</u> SQSEESDLGEGGKKRE ⁷⁰⁶	691S	Glu C	2633.24
⁶⁸³ GQFVVLSSSQ <u>p</u> SEEpSDLGEGGKKRE ⁷⁰⁶	693S, 696S	Glu C	2713.24

^a“p” denotes phosphorylation, asterisk, “*” denotes carboxyamidomethylation.

^b“‡” denotes sequence regions where single residue is known to be phosphorylated between the residues underlined. Phosphorylation on specific residue on those regions cannot be confirmed.

^crepresents digestion by multiple proteases. Trp, Chymo and Glu C correspond to the proteases, trypsin, chymotrypsin, and Glu C, respectively.

Table 2

Phosphorylation Sites Identified within APPL1 by LTQ-Orbitrap-MS

Peptide ^a	Sequence Position ^b	Protease ^c	[M+H] ⁺ (m/z)	Mass error (ppm)
³⁶⁷ IC* <u>TINN</u> p SKQIYLSNPETAARVNQSAL ³⁹⁵	374S	Chymo	3356.66	3.30
³⁹⁰ VNQSALEAVTP <u>SP</u> SFQQR ⁴⁰⁷	‡	Trp	2038.96	-2.45
⁴¹⁵ AGQSRPPTART p SSGSLGSESTNL ⁴³⁸	427S	Chymo	2428.11	-0.62
⁴¹⁸ SRPPTART p SSG p SLGSESTNL ⁴³⁸	427S, 430S	Chymo	2251.96	0.93
⁴¹⁸ SRPPTART p SSGSLGSESTNL ⁴³⁸	427S	Chymo	2171.99	1.10
⁴⁵¹ TPIQFD Il pSPVC*EDQPGQAKAF ⁴⁷²	459S	Chymo	2541.17	0.08
⁴⁵⁶ D Il pSPVC*EDQPGQAKAF ⁴⁷²	459S	Chymo	1954.91	-1.64
⁴⁵⁷ Il pSPVC*EDQPGQAKAF ⁴⁷²	459S	Chymo	1954.86	0.33
⁶⁶⁹ LIAASSRPNQASSEGQFVVL <u>SSSQ</u> SEESDLGEGGK ⁷⁰³	‡	Trp	3631.68	-3.71
⁶⁸³ GQFVVLSS p SQSEESDLGEGGKKRE ⁷⁰⁶	691S	Glu C	2633.21	-0.46
⁶⁸³ GQFVVLSS p SQSEESDLGEGGKKRESE ⁷⁰⁸	693S	Glu C	2849.28	5.58
⁶⁸³ GQFVVLSS p SQSEESDLGEGGKKRE ⁷⁰⁶	693S	Glu C	2633.21	-0.57
⁶⁸⁶ VVLSS p SQSEESDLGEGGKKRE ⁷⁰⁶	691S	Glu C	2301.06	0.13
⁶⁸⁶ VVLSS p SQSEESDLGEGGKKRE ⁷⁰⁶	693S, 696S	Glu C	2381.03	-1.89

^a“p” denotes phosphorylation, asterisk, “*” denotes carboxyamidomethylation.

^b“‡” denotes sequence regions where single residue is known to be phosphorylated between the residues underlined. Phosphorylation on specific residue cannot be confirmed.

^c represents digestion by multiple proteases. Trp, Chymo and Glu C correspond to the proteases, trypsin, chymotrypsin, and Glu C, respectively.

Table 3

Comparison of Peptide Sequence Surrounding Identified Phosphorylation Sites in APPL1

Site	Position	Homologues	Peptide Sequence
‡ 97/98S	92-119	Human	VIDEL <u>SS</u> CHAVLSTQLADAMMFITQFK
		Rat	VIDELSSCHAVLSTQLADAMMFISQFK
		Mouse	-----
374S, 378Y	367-395	Human	ICTINNISKQIYLSNPETAARVNQSAL
		Rat	ICTINNISKQIYLSNPETAARVNQSAL
		Mouse	ICTINNISKQIYLSNPETAARVNQSAL
‡ 401/403S	390-407	Human	VNQSALEAVTP <u>SP</u> SFQQR
		Rat	VNQSALEAVTPSPSFQQR
		Mouse	VNQSALEAVTPSPSFQQR
427S, 430S	418-438	Human	SRPPTARTSSSGSLGSESTNL
		Rat	SRPPTARTSSSGSLGSESTNL
		Mouse	SRPPTARTSSSGSLGSESTNL
459S	451-472	Human	TPIQFDIISPVCEDQPGQAKAF
		Rat	TPIQFDIISPVCEDQPGQAKAF
		Mouse	TPIQFDIISPVCEDQPGQAKAF
491S	479-505	Human	TNPFGESGGSTKSETEDSILHQLFIVR
		Rat	TNPFGESGGSTKSETEDSILHQLFIVR
		Mouse	TNPFGESGGSTKSETEDSILHQLFIVR
604Y	595-614	Human	SESNLSSVCYIFESNNEGEK
		Rat	SESNLSSVCYIFESNNEGEK
		Mouse	SESNLSSVCYIFESNNEGEK
689S, 691S, 693S, 696S	669-703	Human	LIAASSRPNQASSEGQFVVLSSSQSEESDLGEGGK
		Rat	LIAASSRPSQSGSEGQ-LVLSSSQSEESDLGEEGK
		Mouse	LIAASSRPNQAGSEGQ-LVLSSSQSEESDLGEEGK
‡ 689/690/691S	669-703	Human	LIAASSRPNQASSEGQFVVL <u>SS</u> SQSEESDLGEGGK
		Rat	LIAASSRPSQSGSEGQ-LVLSSSQSEESDLGEEGK
		Mouse	LIAASSRPNQAGSEGQ-LVLSSSQSEESDLGEEGK

‡ denotes sequence regions where single residue is known to be phosphorylated between the residues underlined. Phosphorylation on specific residue on those regions cannot be confirmed.

Table 4

Putative Kinases for Identified APPL1 Phosphorylation Sites

Sequence Position	MS-based method	Putative Kinases ^a
401 S	LTQMS/ LTQ-Orbitrap	cell division cycle 2 glycogen synthase kinase 3 cyclin dependent kinase 5
427 S	LTQ-Orbitrap	ribosomal s6 kinase Akt protein kinase A
430 S	LTQ-Orbitrap	protein kinase C casein kinase 1
491 S	LTQMS	casein kinase 2
689 S	LTQ-Orbitrap	cell division cycle 2
691 S	LTQMS/ LTQ-Orbitrap	casein kinase 2 DNA protein kinase cell division cycle 2
693 S	LTQMS/ LTQ-Orbitrap	casein kinase 1
696 S	LTQMS/ LTQ-Orbitrap	casein kinase 2 casein kinase 1

^aKinases predicted to phosphorylate the indicated sites using NetPhosK 1.0 (0.5 threshold) and Scansite (Medium Stringency).