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Mass spectroscopic phosphoprotein mapping of Ral binding protein 1 (RalBP1/Rip1/RLIP76)

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Abstract

RalBP1, a multifunctional protein implicated in cancer cell proliferation, radiation and chemoresistance, and ligand dependent receptor internalization, is upregulated in bladder cancer and is a downstream effector of RalB, a GTPase associated with metastasis. RalBP1 can be regulated by phosphorylation by protein kinase C (PKC). No studies have comprehensively mapped RalBP1 phosphorylation sites or whether RalB affects these. We identified 14 phosphorylation sites of RalBP1 in human bladder carcinoma UMUC-3 and embryonic kidney derived 293T cells. The phosphorylated residues are concentrated at the N-terminus. Ten of the first 100 amino acids of the primary structure were phosphorylated. Nine were serine residues, and one a threonine. We evaluated the effect of RalB overexpression on RalBP1 phosphorylation and found the largest change in phosphorylation status at S463 and S645. Further characterization of these sites will provide novel insights on RalBP1 biology, its functional relationship to RalB and possible avenues for therapeutic intervention.

Keywords: RalBP1; Phosphorylation; Ral GTPase

RalBP1, a multifunctional protein, plays a role in cell signaling [1–5], ligand dependent receptor internalization [6–8], and xenobiotic defense mechanisms, putatively as a membrane bound ATP dependent pump [9,10]. The latter is particularly important since RalBP1 plays a role in cancer cell chemoresistance [11]. Originally, this protein was cloned as a GTP-dependent interaction partner to RalA, a small GTPase downstream of Ras with importance for human carcinogenesis [2]. RalBP1 also binds directly to RalB [12], a homologous protein associated with cancer metastasis [3].

RalBP1 shares sequence similarity with GAP proteins capable of activating the GTPase activity of members of the Rho/Rac family of GTPases. RalBP1 activates the GTPase activity of Cdc42 and, to a lesser extent, Rac1 but not RhoA, Ras, or Ral [13]. Since RalBP1 directly binds RalB and may affect Rac1 and Cdc42 activity, two proteins involved in cell migration [14], RalBP1 may be critical in tumor invasion and metastasis. Moreover, since RalBP1 is specifically expressed in bladder cancer more than other human tumors [15], the functional relevance of this protein may be even more important in bladder cancer.

Despite the important regulatory role of phosphorylation in cell signaling, little is known about RalBP1. Recent reports on RalBP1 regulation by Protein Kinase C alpha (PKCalpha) sequence analysis indicated four putative PKCalpha phosphorylation sites [16]. In vitro analysis of the four amino acids (S118, T297, S353, and S509) indicates that T297 is by far the most abundantly phosphorylated site [17]. PKC knockdown does not affect doxorubicin sensitivity of mouse embryonic fibroblasts from a RalBP1$^{-/-}$ mouse but increases that of RalBP1$^{+/+}$ derived cells [18], further indicating a regulatory role of PKC for RalBP1.

In summary, RalBP1 is an important signaling intermediary in cancer, affecting cell migration and metastasis and chemotherapeutic resistance. We sought to comprehensively map the phosphorylation sites of RalBP1 using mass

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spectroscopic analysis and determine whether RalB, a major upstream binding partner of RalBP1, affects these.

Materials and methods

Cell culture, cDNA constructs, and transfection procedures. UMUC-3 cells were cultured as described [19]. 293T cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM with 2 mmol/L L-glutamine, 4.5 g/L D-glucose, and 10% fetal bovine serum (FBS). Plasmid transfections were performed with FuGENE according to manufacturer's instructions (Roche, Basel, Switzerland). Full length N-terminally tagged HA-RalBP1 was obtained from GeneCopoeia (Germantown, MA, USA). Ral constructs have previously been described [19].

Western blots. Cells were harvested in modified RIPA lysis buffer (50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% Igepal) and supplemented with protease and phosphatase inhibitor (Sigma, St. Louis, MO, USA). The lysates were incubated for 20 min before un-dissolved residues were spun down. Antibodies were purchased from the following manufacturers: β-Tubulin: Calbiochem (Darmstadt, Germany); HA.11: Covance (Denver, PA, USA); Phospho-threonine and Phospho-Tyrosine (P-Tyr-100): Cell Signaling (Beverly, MA, USA); RalBP1 Abnova (Taipei City, Taiwan).

Immunoprecipitation and mass spectrometry (MS) analysis. 18 μg of DNA and 56 μl FuGene was used to transfect a 70% confluent 150 mm plate and incubated for 24 h before harvest in 2 ml lysis buffer (see above) including protein and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Lysates were incubated for 2 h with covalently conjugated anti-HA agarose beads (Sigma, St. Louis, MO, USA) and washed in lysis buffer four times. Precipitates were analyzed by Western blotting as described above. For MS analysis, precipitates were separated by SDS–PAGE and stained with colloidal Coomassie blue stain. Briefly, gels were treated 2×15 min in fix solution (10% Acetic acid, 40% Methanol) before overnight staining in Colloidal Coomassie Stain solution (80 ml stock solution (1.5 mM Coomassie Brilliant Blue R250, 3% v/v H_3PO_4, 950 mM $(NH_4)_2SO_4$) diluted with 20 ml Methanol). The protein band migrating at the appropriate size as determined by comparison to Kaleidoscope weight standards (BioRad, Hercules, CA, USA) was excised. The gel piece was transferred to a siliconized tube, washed and destained in 200 μl 50% methanol overnight and subsequently dehydrated in acetonitrile, rehydrated in 30 μl of 10 mM dithiolthreitol in 0.1 M ammonium bicarbonate and reduced at room temperature for 30 min. DTT was removed and the sample alkylated in 30 μl 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The reagent was removed, the gel pieces dehydrated in 100 μl acetonitrile, then rehydrated in 100 μl of 0.1 M ammonium bicarbonate. A final dehydration step was performed in 100 μl acetonitrile, the acetonitrile removed and the pieces completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/μl (Trypsin and Arg-C) in 50 mM ammonium bicarbonate buffer at 4 °C for 10 min. Any excess enzyme solution was removed and 20 μl 50 mM ammonium bicarbonate added. The sample was digested overnight at 37 °C and the peptides extracted from the polyacrylamide in two 30 μl aliquots of 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to 15 μl for MS analysis.

The LC–MS system was a Finnigan LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 μm id Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. 0.5–5 μl volumes of extract were injected and peptides eluted from the column by acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 μl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. This analysis produces approximately 2000 CAD spectra of ions ranging in abundance over several orders of magnitude. Not all CAD spectra are derived from peptides.

The data were analyzed by database searching using the Sequest search algorithm against Human International Protein Index (IPI) and then RalBP1.

Computational analysis of phosphorylation sites. Computational analysis was performed using the Eukaryotic Linear Motif (ELM) Resource for Functional Sites in Proteins available at http://elm.eu.org/.

Results

RalBP1 is phosphorylated at threonine residues and this is not altered by serum deprivation

To evaluate the precipitation and detection of RalBP1 protein, the expression construct was transfected into 293T cells and harvested the following day. RalBP1 was detected by anti-HA or anti-RalBP1 antibody in the whole cell lysate (Fig. 1). Enough antigen was readily precipitated

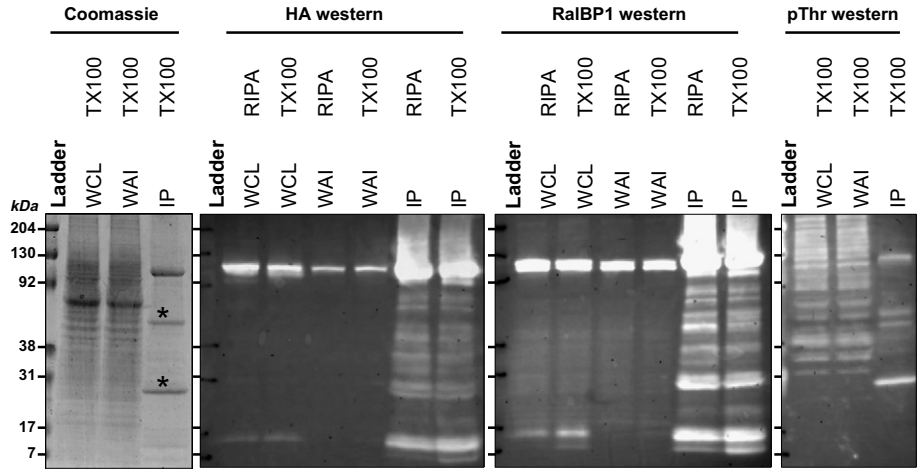
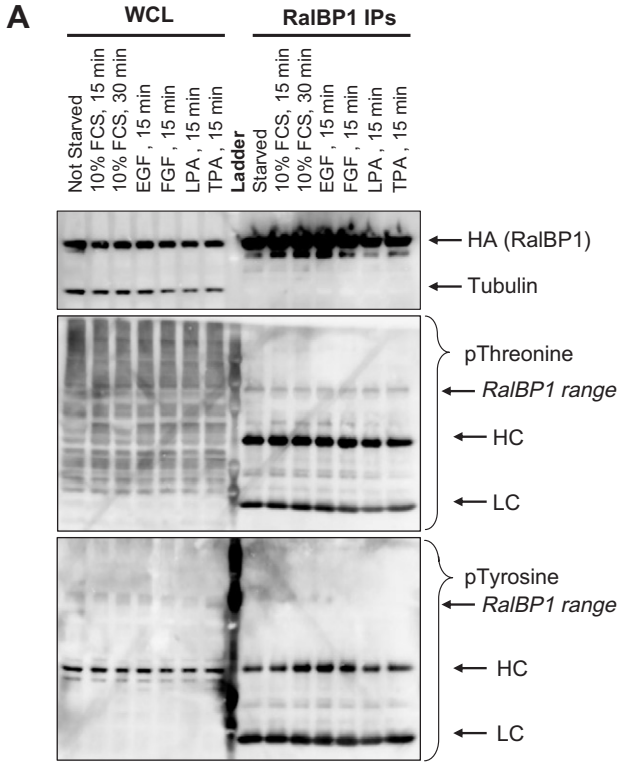


Fig. 1. RalBP1 was precipitated from 293T cells transiently transfected with RalBP1-HA and detected directly with Coomassie blue staining or indirectly through anti-HA antibodies, anti-RalBP1 antibodies, or anti-phospho-threonine (pThr) antibodies. Two solubilization protocols were evaluated, RIPA buffer or Triton X-100 containing buffer. Antigen was detected before precipitation in whole cell lysate (WCL) and in whole cell lysate after precipitation (WAI). The precipitate was boiled and loaded (IP), and enrichments and purification were observed as depicted. (*) Antibody fragments.

by the HA antibody to present a clear band in a Coomassie blue stained gel. No major contaminating bands were obvious. Western blot with anti-phospho-Tyrosine (data not shown) and anti-phospho-threonine (Fig. 1) antibodies indicated that RalBP1 is phosphorylated at threonine residues but not at Tyrosine residues. To examine how phosphorylation was regulated by extracellular stimuli, several common phosphorylation cues were investigated. Cells were transfected as previously described and deprived of serum in their growth media for 24 h, then stimulated with 10% FCS, 50 ng/ml EGF, 50 ng/ml FGF, 5 μM LPA or 200 nM TPA for 15 min. Cells were harvested, HA-RalBP1 was precipitated and precipitates were analyzed by SDS–PAGE with subsequent Western blotting. No significant changes in phosphorylation levels between serum deprived and stimulated cells was detected utilizing anti-phospho-threonine antibodies (Fig. 2A).

RalBP1 is primarily phosphorylated at the N-terminus

To determine the residues phosphorylated on RalBP1, 293T cells were transfected with HA-RalBP1 and precipitated as described. A strong Coomassie blue stained band at the correct molecular weight was excised from the gel and examined by mass spectroscopy. This detected RalBP1-derived peptides covering 60% of the total protein



B

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1   mtecflppts spsehrrveh gsgltrtpss eeisptkfpg lyrtgepspp hdilheppdv
61  vsddekdhgk kkgkfkkkek rtegyaafqe dssgdeaesp skmkrskgih vfkkpsfskk
121 kekdfkikek pkeekhkeek hkeekhkekk skdltaadvv kqwkekkkkk kpiqepevpq
181 idvpnlkpif gipladaver tmmydgirlp avfrecidyv ekygmkcegi yrvsgikskv
241 delkaaydre estnledyep ntvasllkqy lrdlpenllt kelmprfeea cgrttetekv
301 qefqrllkel pecnyllisw livhmdhvia keletkmniq nisivlsptv qisnrvlyvf
361 fthvqelfgn vvlkqvmkpl rwsnmatmpt lpetqagike eirrqeflln clhrdlqggi
421 kdlskeerlw evqriltalk rklreakrqe cetkiaqeia slskedvske emneneevin
481 illaqeneil teqeellame qflrrqiase keeierlrae iaeiqsrqqh grseteeyss
541 esesesedee elqiiledlq rqneeleikn nhlnqaihee reaiielrvq lrllqmqrak
601 aeqqaqedee pewrggavqp prdgvlepka akeqpkagke pakpspsrdr ketsi
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Fig. 2. (A) Western blot showing RalBP1 immunoprecipitation (IP) of cells transfected with RalBP1-HA, serum-starved for 24 h and stimulated as indicated. Analysis of whole cell lysates (WCL) indicates that equal amounts of RalBP1-HA were expressed in cells. Comparable levels of RalBP1-HA were precipitated, as indicated by HA-Western blot. No differences in phosphorylation were detected by phospho-threonine or Tyrosine specific antibodies. Heavy and light chains (HL and LC) are marked accordingly. (B) Primary sequence of RalBP1 showing peptides detected in mass spectroscopic analysis (yellow). Four hundred and six of 655 amino acids, 62%, were covered. Fourteen sites were detected (bold). Two sites were ambiguous. It was unclear whether S116 or S118 was phosphorylated or whether it was S252 or T253 that was modified. Twenty-nine of the 41 serine residues, 17 of 26 threonine, and 8 of 12 Tyrosine residues were covered. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Phosphorylated amino acid residues in RalBP1 and computationally derived putative kinases and binding proteins

Cell line		Putative phosphorylation site by ELM	
293T	UMUC-3	Potential kinase	Potential binding site
S11	S11	MAPK, CK2	WW4
T27	T27	MAPK	WW4
S29	S29	CK2	14-3-3
S30	S30		
S34	S34	CDK, MAPK	WW4
S48	S48	MAPK	WW4
S62	S62	CK2	
S92	S92	CK2	
S93	S93		
S99	S99	CDK, MAPK	WW4
S116 or S118[a]	S116 or S118	PKC (S118)[b]	
S252 or T253[a]	S252 or T253		
S463	S463		
S645	S645		

[a] Located too close to determine which was phosphorylated.
[b] S118 was previously reported [17].

Fig. 3. In three different regions several peptides were phosphorylated at multiple sites. Detected phosphorylations are indicated in red; peptides detected are written out. In the first region, only one peptide was detected with phosphorylation at T27. Most S29, S30, and S34 phosphorylation patterns were detectable, with one exception. S29 was never phosphorylated together with S34 alone. In the second region, pS48 was only detected in peptides with pS62 peptides. No peptides were detected without S62 phosphorylation. The third region where more than one residue was detected to be phosphorylated contained the S92, S93, S99 sites. No peptides without S92 and S93 phosphorylation were detected. These observations are identical between the UMUC-3 and 293T cell lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2B). Fourteen separate phosphorylated residues were found (Table 1). Ten of the 14 residues are located within the first 100 amino acids of the protein and 13 of the 14 are serine residues. To verify these results, an identical experiment was carried out in the bladder cancer cell line UMUC-3. All 14 residues detected in 293T were also found in UMUC-3 (Table 1).

Several phosphorylated sites within the first 100 amino acids were observed within the same peptide. Fig. 3 shows the different distinct peptides detected with more than one phosphorylated residue. Only peptides covering the T27-S34 phosphorylation sites were also void of phosphorylation modifications. Only the S62 and S92/S93 sites were not detected in an unmodified form. Phosphorylation at T27 was not detected in peptides without pS30. Phosphorylation at S48 (pS48) was not detected in peptides without pS62. pS99 was not detected in peptides not phosphorylated at S92 and S93, and these two residues were always simultaneously modified. The same observations were made in both cell lines.

Computational analysis of putative kinases responsible for RalBP1 phosphorylation

To identify kinase candidates and putative kinase-regulated binding sites responsible for the phosphorylation sites, a domain analysis was carried out utilizing the Eukaryotic Linear Motif Resource for Functional Sites in Proteins [20]. Mitogen Activated Protein Kinases (MAP Kinases), Cyclin Dependent Kinases (CDK), and Casein Kinase 2 (CK2) were among the identified kinase candidates. One putative 14-3-3 biding site at residue S29 and four phosphorylated WW4 domains were detected (Table 1).

RalB overexpression increases RalBP1 phosphorylation at the C-terminus

To determine whether RalB overexpression influences the phosphorylation status of RalBP1, a similar experiment to the one above was carried out. Wild type RalB or control vector was co-transfected with HA-RalBP1 in 293T cells. RalB expressed at high levels and RalBP1 could be readily precipitated and detected. Twelve phosphorylation sites were again detected. Peptides for T27 and S252/T253 were not detected in this data set. While the N-terminus is heavily phosphorylated, none of these sites showed significant change in phosphorylation status with RalB. The Ral interaction domain is much closer to the C-terminus, where the largest change in phosphorylation status was observed at sites S463, merely ten residues from the C-terminus at S645. These sites show a 3.5- and a 5.5-fold higher phosphorylation level in cells overexpressing RalB compared to vector control cells. All other sites show smaller changes between RalB-overexpressing cells and controls and are deemed too small to be significant (Fig. 4).

Discussion

To broaden the understanding of the multifunctional protein RalBP1 we undertook a systematic approach to identify and analyze its post-translational modification.

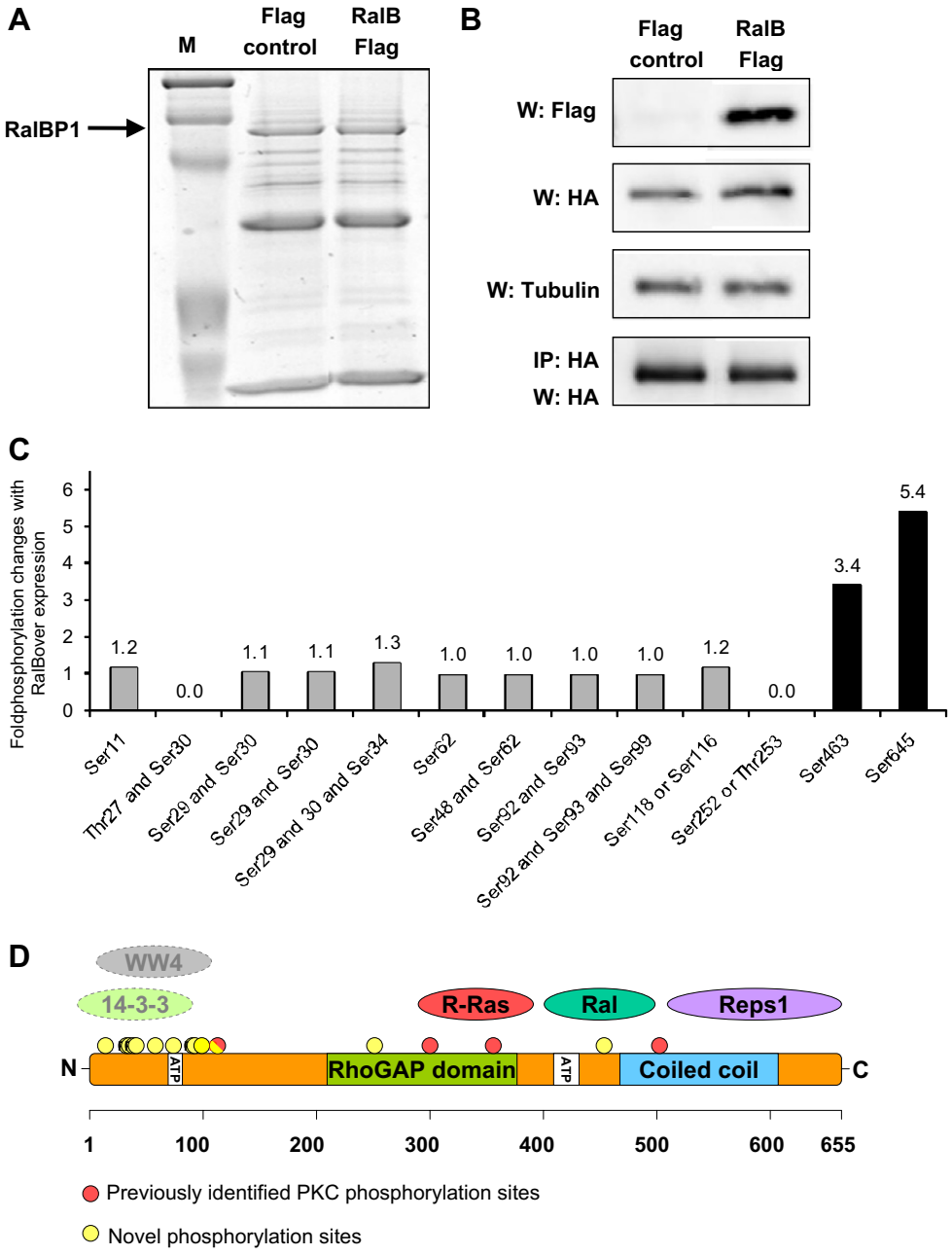

Fig. 4. (A) RalBP1 was precipitated from 293T cells transiently transfected with RalBP1-HA with Flag control vector or RalB-Flag and detected with Coomassie blue staining. (B) Western (W) blot or IP for Flag or HA indicating expression of RalBP1-HA and RalB-Flag in corresponding samples. (C) Phosphorylation changes in RalBP1-HA in 293T cells overexpressing wild type RalB. Only sites S463 and S645 show significant change in phosphorylation levels between mock and RalB-transfected cells. (D) RalBP1 structural domains include RhoGAP and the coiled coil at aa 207–373 and 469–610, respectively (http://elm.eu.org/). ATP binding sites (aa 65–80 or 415–448) [16]. Putative interaction partners are schematically depicted [25,26]. Previously suggested PKC phosphorylation sites are in red and include 118S, 297T, S353, and S509 [17]. Phosphorylation sites described here are in yellow and include S11, T27, S29, S30, S34, S48, S62, S92, S93, S99, S116 or S118, S252 or T253, and S463. S118 is indicated as half red and half yellow. Putative binding proteins 14-3-3 and WW4-binding proteins shown in computationally assigned locations.

None of the previously identified PKC phosphorylation sites S118, T297, S353, and S509 [17] were conclusively identified. In our data only S118 was tentatively verified. It was not possible to determine whether S116 or S118 was phosphorylated in that specific peptide. S509 and S353 were unmodified in our cell lines. The most strongly modified PKCalpha residue, T297 [17], was not covered by our analysis.

The primary sequence was further analyzed in silico by publicly available protein domain identification algorithms for the potential impact of phosphorylation or the potential kinase that modified that particular site. The first molecule identified as a phosphorylation-dependent interaction partner was 14-3-3. The consensus site [RHK][STALV].[ST].[PESRDIF] matches the S30 site 26-RTPSSE-31 and indicates that this might be an important interaction site

for this family of signaling molecules. 14-3-3 molecules are the archetypical scaffold proteins that organize signaling complexes and have been found to interact with more than 200 different molecules [21]. 14-3-3 modifies the function of its targets by changing their activity, intracellular localization, and the composition of larger complexes that they associate with. They exert these functions mainly via phospho-serine/threonine dependent protein–protein interactions. The identification of a 14-3-3 interaction motif in the N-terminus of RalBP1 is exciting, since it implies that this part of the protein may have a crucial role in determining its molecular context.

WW4 domains have a small recognition motif consisting of a phosphorylated serine or threonine followed by a proline. The S11, S34, S48, and S99 motifs are all potentially WW4 binding motifs [22,23]. WW4 domains are similar to 14-3-3 interaction domains, with a protein–protein interaction domain dependent on phosphorylation. Interestingly, none of these sites are constitutively phosphorylated in the N-terminus (Fig. 3) which may indicate that phosphorylation of these sites could modulate RalBP1 function.

Sites for Cyclin dependent Kinases, Casein Kinases, and praline-directed Mitogen Activated Protein Kinases all are integral in the control of the cell cycle. These observations provide little information about the potential roles for these modifications of RalBP1 but do provide a list of plausible suspects that could be revisited as more data becomes available. Although the MAPK sites are at hand, the consensus sites [ST]P have little significance if other docking domains are not at hand. No specific docking domains for MAP Kinases were found in the protein, although several Lysine and Arginine-rich domains were identified, previously noted as important for MAPK substrate docking that can be located in a distant region of the protein [24].

Some sites were close enough to determine whether they were modified in certain patterns. These patterns might indicate that the phosphorylation of one site depends on another site. For instance S62 phosphorylation might be mandatory for S48 to become phosphorylated. However, this might also indicate that S62 is constitutively phosphorylated, whereas S48 is regulated by an as-yet-unknown mechanism. The same arguments might be made for the other sites depicted in Fig. 3. The analysis of these regions does, however, indicate which sites are more frequently unmodified compared to neighboring sites.

Finally, cells overexpressing RalB and mock transfected cells were compared. In this analysis changes at two sites were detected. Especially exciting is the significant change in phosphorylation at site S463 as this is within the smallest required sequence [25] to bind Ral to RalBP1 (Fig. 4). The largest caveat is the low level of phosphorylation at this site, which makes this observation less sure. However, our previous studies showed that less than 5% of wild type Ral is bound to RalBP1 in even the most favorable situations [19]. In a situation where Ral binding to RalBP1 promotes phosphorylation, or RalBP1 phosphorylation at this site allows binding, a low level of phosphorylation could be expected. The observed phosphorylation changes at the S645 site are robust, and observed on more peptides, which solidifies the observation. S645 is located very close to the C-terminus and could have bearing on larger tertiary structure modifications of the protein and cellular localization, or involve other sites, like the binding domain for Ral.

In summary, we present the first systematic analysis of RalBP1 phosphorylation and describe several novel phosphorylation sites. These studies identify the N-terminus of the protein as the region most strongly modified by phosphorylation. The N-terminus has not yet been designated for any specific function, and strong post-translational modification in this area is therefore intriguing. Finally, we have tantalizing evidence of RalB-induced phosphorylation. These preliminary data open up an exciting area of study that will shed light on the regulation of RalBP1 downstream signaling events.

Acknowledgments

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