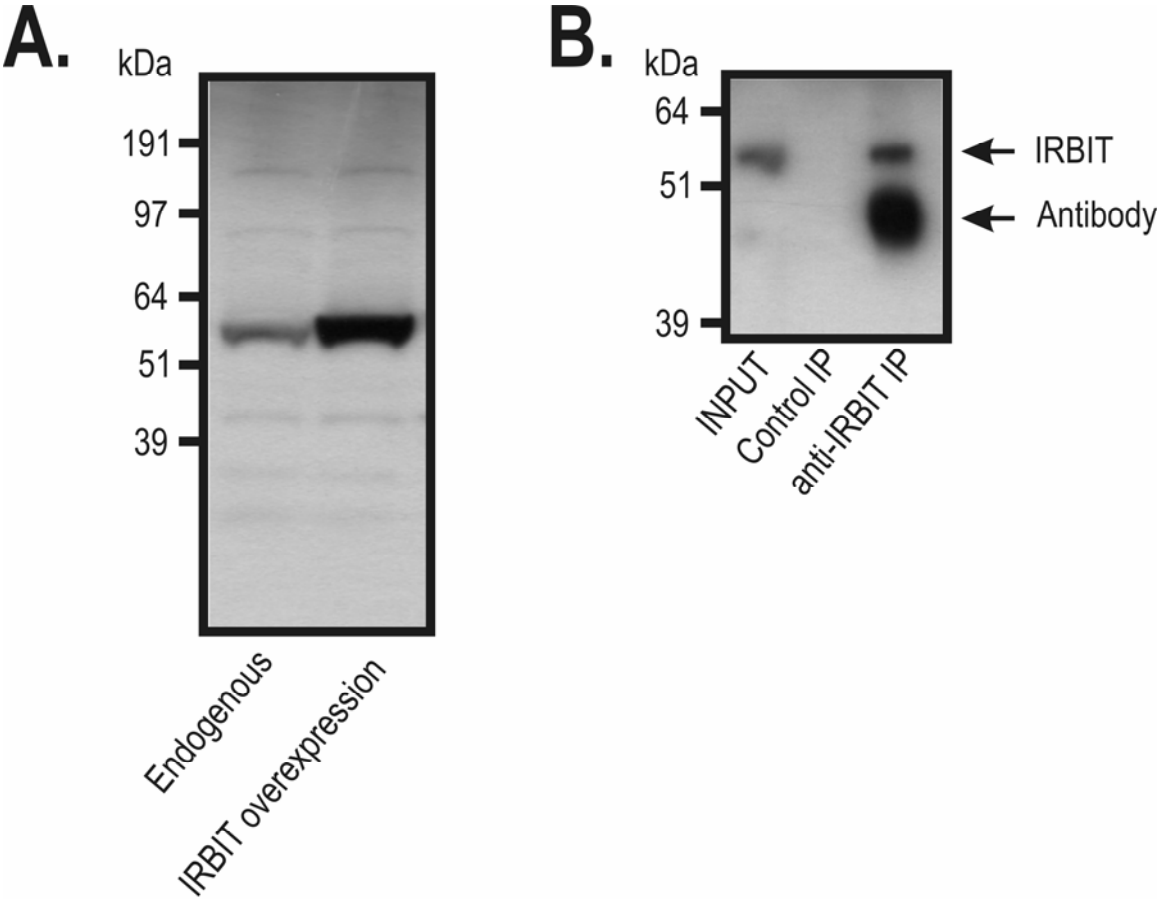
SUPPLEMENTAL DATA

Supplemental data Figure S1.

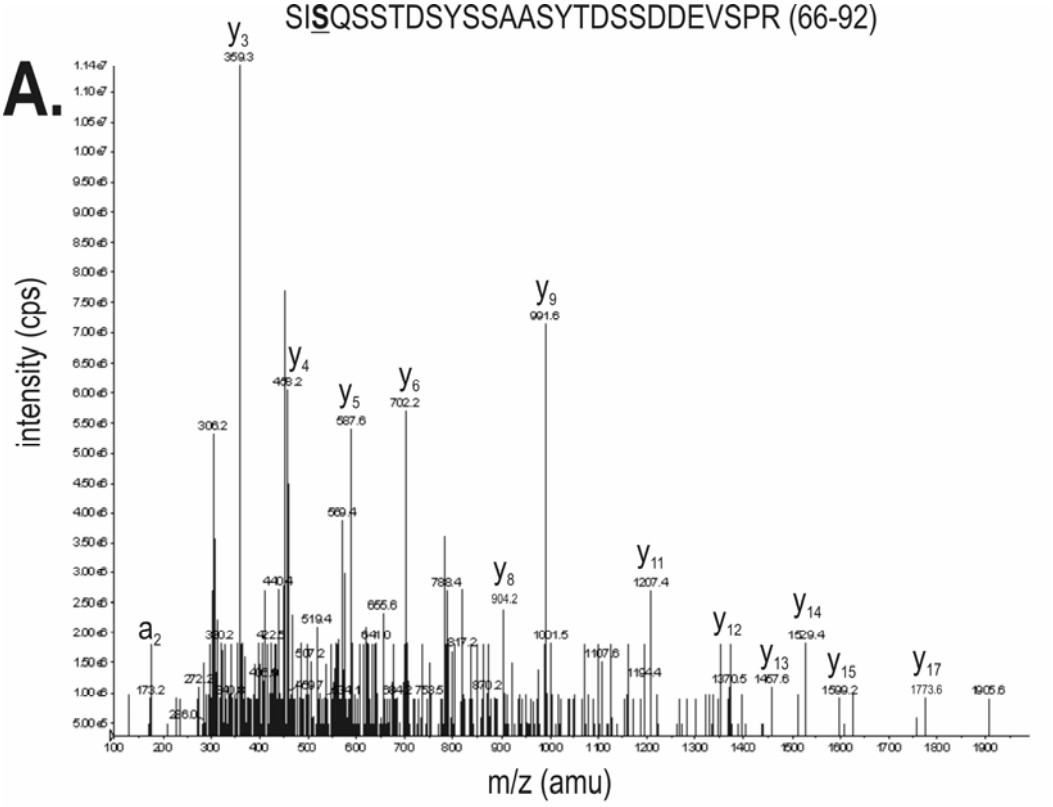


Supplemental data Figure S1: Validation of the anti-IRBIT antibody.

A, COS-1 cells were transfected with pTRACER-IRBIT. The whole cell lysate of the untransfected COS-1 cells (for endogenous IRBIT) or transfected cells (for overexpressed IRBIT) were analyzed by immunoblotting using the anti-IRBIT antibody.

B, COS-1 cells were lysed (INPUT) and endogenous IRBIT was immunoprecipitated using the anti-IRBIT antibody (anti-IRBIT IP). Immunoprecipitation in the absence of the antibody was used as a control (control IP). The positions of IRBIT and the immunoglobulin heavy chain of the anti-IRBIT antibody are indicated.

Supplemental data Figure S2

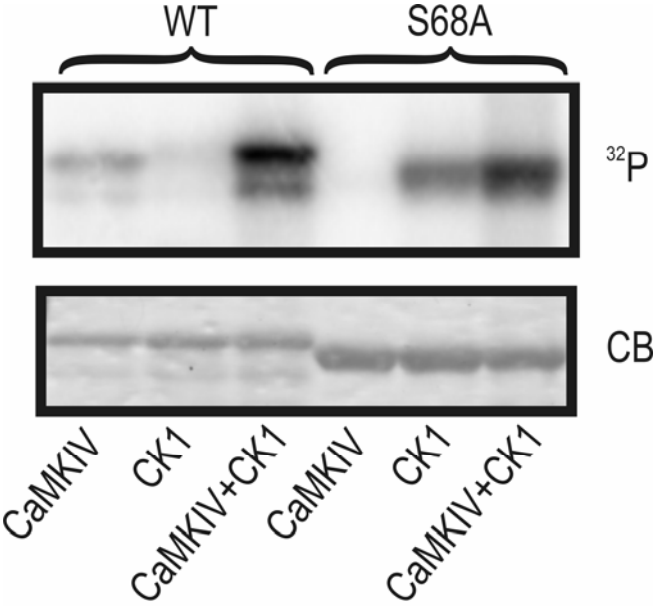


Supplemental data Figure S2: Mass-spectrometric analysis of IRBIT phosphorylated by PKD.

Purified GST-IRBIT-[1-104] was added to a reaction mixture containing 50 mM glycylglycine pH 7.5, 50 µg/ml BSA and 20 µg/ml PKD. Reaction was started by addition of a Mg^{2+}-ATP mixture (1 mM $MgCl_2$, 1 mM ATP) and reaction tubes were incubated for 2 h at 30 °C. Reaction was stopped by addition of 10 mM EDTA and incubation on ice. The samples were precipitated with 10 % trichloroacetic acid and the acetone-washed pellet was digested overnight with 250 ng trypsin in 200 mM ammonium bicarbonate. The resulting peptide mixture was analysed by a nano LC-MS/MS approach performed on a Dionex Ultimate capillary liquid chromatography system coupled to an Applied Biosystems 4000 QTRAP mass spectrometer. Peptides were separated on a PepMap C18 column developed with a 30 min linear gradient (0.1 % formic acid - 6 % acetonitrile - water to 0.1 % formic acid – 40 % acetonitrile – water). After pinpointing m/z values of potentially phosphorylated species of IRBIT-[66-92] by a precursor 79 (-) ion scan, we selected these ions for on-line collision-induced dissociation (CID) in a product (+) ion scan to determine the phosphorylated residue(s).

A product ion spectrum of the mono-phosphorylated form of IRBIT-[66-92] is shown. An a_2 ion (m/z 173) which corresponds to non-phosphorylated IRBIT-[66-67], together with both an a_4 ion (m/z 468) and a b_4 ion having lost H_3PO_4 (m/z 398), corresponding to mono-phosphorylated IRBIT-[66-69], were found. These ions pinpointed Ser-68 as the phosphorylated residue. We could not detect a di-phosphorylated form of IRBIT-[66-92], indicating that Ser-68 was the only phosphorylated residue in this sample.

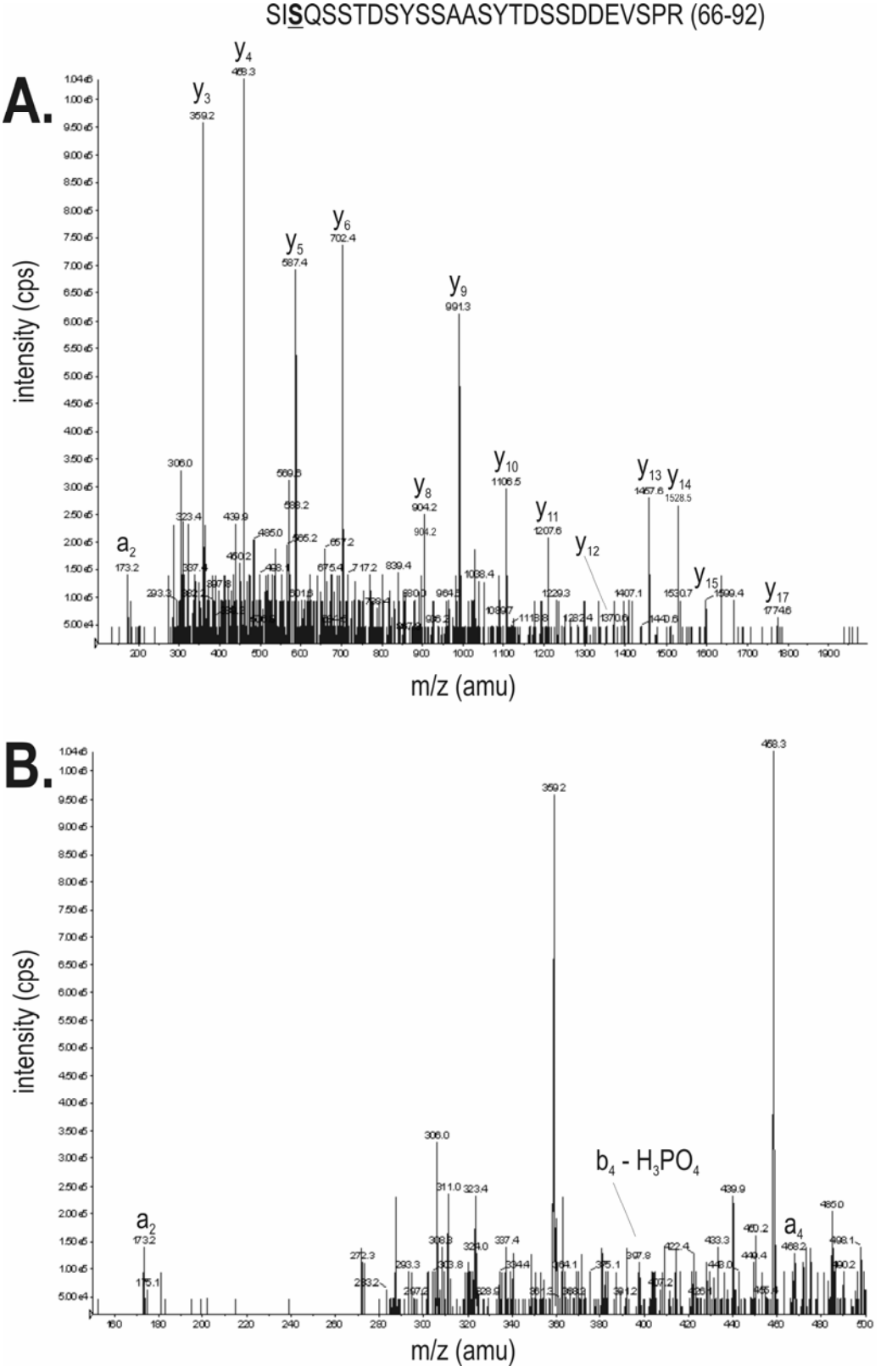
Supplemental data Figure S3



Supplemental data Figure S3: Phosphorylation by CaMKIV on Ser-68 allows for subsequent phosphorylation by CK1.

Purified GST-IRBIT-[1-104] (WT) or GST-IRBIT-[1-104]-[S68A] (S68A) was added to a reaction mixture containing 50 mM glycylglycine pH 7.5, 50 µg/ml BSA, and 5 µg/ml CaMKIV (Invitrogen Ltd) and/or 30 µg/ml CK1. The phosphorylation reaction was started by addition of a Mg^{2+}-[γ-^{32}P]ATP mixture giving a final concentration of 2 mM $MgCl_2$ and 0.1 mM ATP (with 0.1 µCi/µl [γ-^{32}P]ATP) in the assay. After 1 h incubation at 30 °C, the reaction was stopped by addition of 10 mM EDTA. Samples were separated on a 10 % Bis-Tris NuPAGE® gel. After staining with Coomassie Blue, radioactivity was detected using phosphoscreens and the STORM 840 PhosphoImager (GE Healthcare). The autoradiogram (indicated as ^{32}P) or Coomassie Blue staining (indicated as CB) from a typical gel are shown (n = 3).

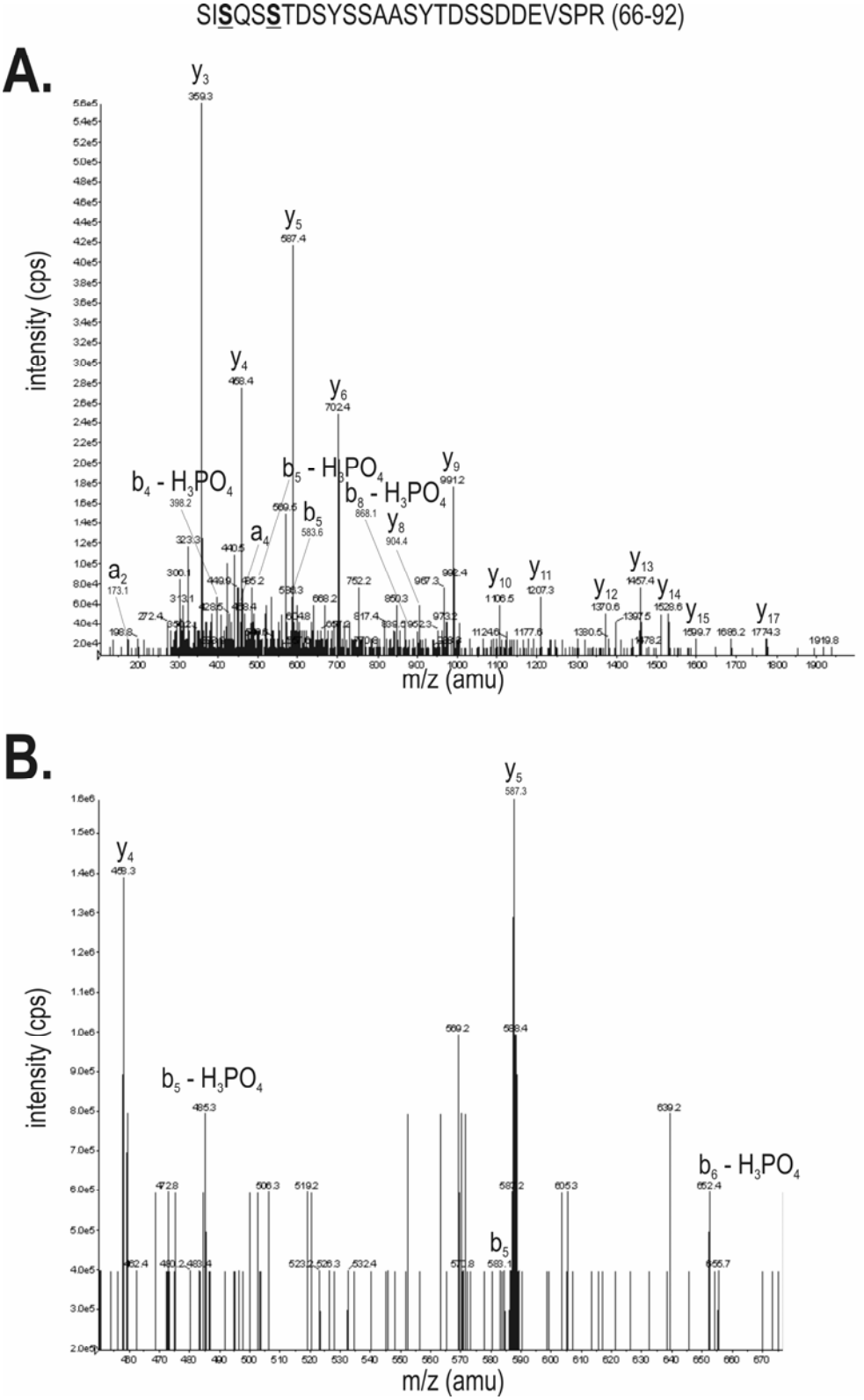
Supplemental data Figure S4



Supplemental data Figure S4: Mass-spectrometric analysis of IRBIT phosphorylated by PKD and CK1 (mono-phosphorylated species).

Sample was treated as described for Figure S2, except that GST-IRBIT-[1-104] was phosphorylated by both PKD and 30 µg/ml CK1. A product ion spectrum of the mono-phosphorylated form of IRBIT-[66-92] is shown. As described for Figure S2, the presence of the ions a_2 (m/z 173), a_4 (m/z 468) and b_4 which lost H_3PO_4 (m/z 398), pinpointed Ser-68 as the phosphorylated residue.

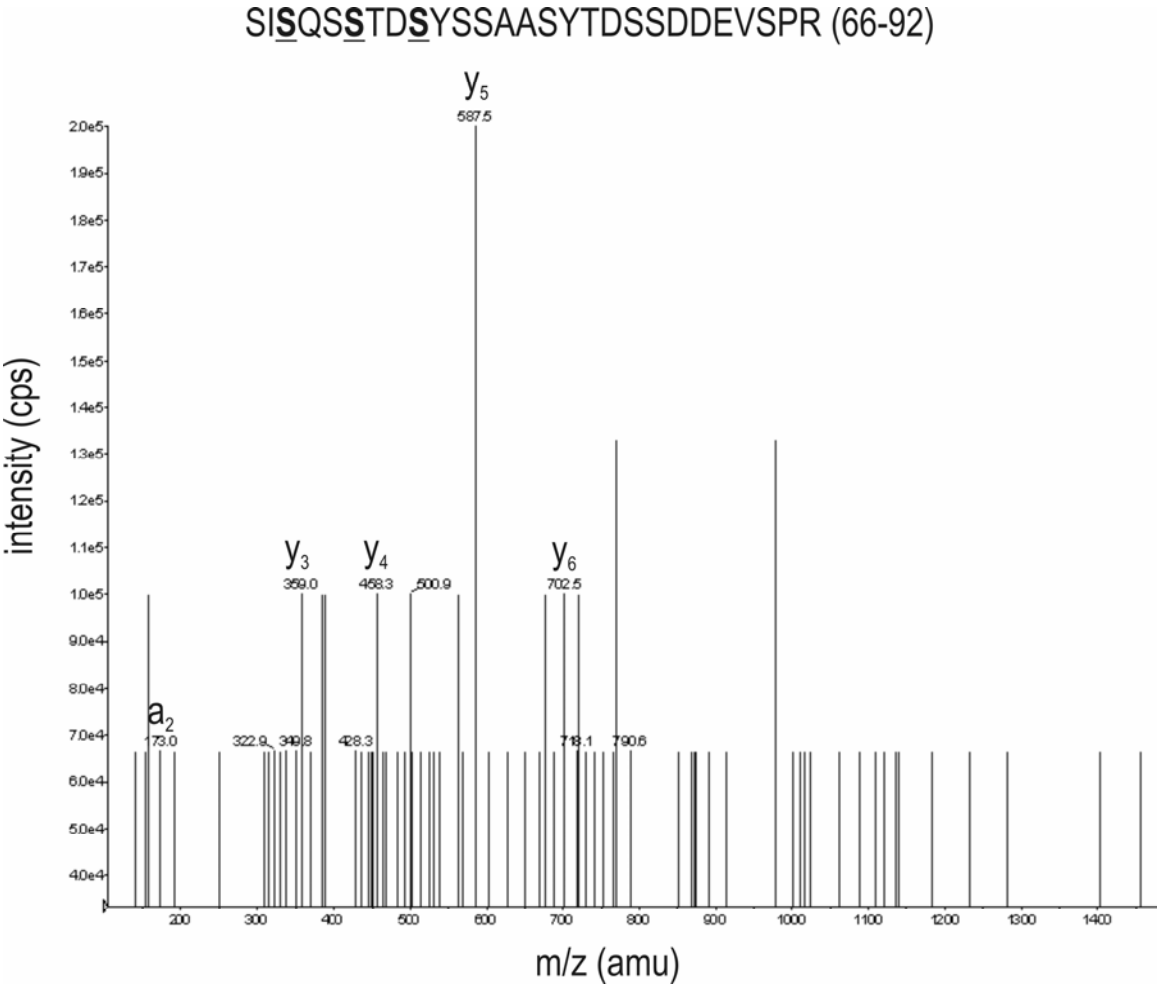
Supplemental data Figure S5



Supplemental data Figure S5: Mass-spectrometric analysis of IRBIT phosphorylated by PKD and CK1 (di-phosphorylated species).

Sample was treated as described for Figure S4. A product ion spectrum of the di-phosphorylated form of IRBIT-[66-92] is shown. The presence of the ions a_2 (m/z 173), a_4 (m/z 468) and b_4 which lost H_3PO_4 (m/z 398), pinpointed Ser-68 as a phosphorylated residue. The presence of both a b_5 ion (m/z 583) and a b5 ion having lost H_3PO_4 (m/z 485), corresponding to mono-phosphorylated IRBIT-[66-70], together with a b_6 ion having lost H_3PO_4 (m/z 652)), corresponding to di-phosphorylated IRBIT-[66-71], demonstrates that Ser-71 is the other phosphorylated residue in this species. The presence of a non-phosphorylated y_{17} (m/z 1774) corresponding to IRBIT-[76-92]) indicated that IRBIT-[76-92] was not phosphorylated.

Supplemental data Figure S6



Supplemental data Figure S6: Mass-spectrometric analysis of IRBIT phosphorylated by PKD and CK1 (tri-phosphorylated species).

Sample was treated as described for Figure S4. A product ion spectrum of the tri-phosphorylated form of IRBIT-[66-92] is shown. In spite of its low abundance, this species produced a product ion spectrum with enough ions to confirm the identity. However, it did not allow us to assign phosphorylation sites.