obtained as described above were diluted in four volumes of immunoprecipitation buffer (Tris-HCl (20 mM), pH 7.5, NaCl (150 mM), sucrose (0.25 M), EGTA (5 mM), EDTA (5 mM), leupeptin (15 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), PMSF (2.5 mM), 0.5% Triton-X, sodium pyrophosphate (10 mM), NaF (25 mM), Na_3VO_4 (5 mM), β-glycerophosphate (50 mM) and levamisole (1 mM)); supernatants were used for immunoprecipitation.

Measurement of phosphatidylinositol-(3,4,5)-trisphosphate. Phosphatidylinositol-(3,4,5)-trisphosphate in unstimulated neutrophils prepared from healthy controls and patients with XLA was measured with an enzyme-linked immunosorbent assay kit in accordance with the manufacturer's instructions (K-2500; Echelon).

Immunofluorescence staining. Cytospin preparations of neutrophils were air-dried and fixed for 10 min with paraformaldehyde in PBS, pH 7.4, then were made permeable for 20 min at −20 °C with acetone, washed, and incubated with the appropriate antibodies. After labeling and washing with 0.2% BSA in PBS, coverslips were mounted with Fluoromount G and the prepared specimens. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Slides were analyzed with a fluorescence microscope (FV10i; Olympus) equipped with Fluoview viewer and review station (Olympus). At least 100 cells were inspected for each slide.

Generation of Hph-1-Btk, Hph-1-Btk mutants, and transduction of recombinant protein into cells. Hph-1–tagged Btk constructs were generated by amplification of a full-length Btk cDNA fragment with the appropriate primers (**Supplementary Table 1a**). After the sequence of each PCR product was verified by DNA sequencing, the fragment was ligated into sites of a pET28b vector (Merck) cleaved by *Xma1* and *Sal1*; the vector has a six-histidine site for protein purification and two tandem Hph-1 sequences for protein transduction. Constructs with deletion of the Tec homology domain, SH3 domain or SH2 domain were generated by mutagenesis with the QuikChange SiteDirected Mutagenesis Kit (Stratagene) and the appropriate primers (**Supplementary Table 1b**). The Hph-1-Gal4 construct has been described[52]. Proteins were induced in BL21 Star competent cells (Novagen) as described[52]. Proteins were treated with Detoxi-Gel Endotoxin Removing Gel (Takara Bio) for elimination of endotoxins and were frozen at −80 °C until further use. Neutrophils (1×10^6 per ml) were incubated for 1 h with 1 μM Hph-1–tagged proteins (80 μg recombinant Hph-1–tagged full-length-Btk was used for 1×10^6 neutrophils for transduction at a concentration of 1 μM) and washed, then ROS production was assayed.

Btk-precipitation assay. Lysates of neutrophils from healthy controls were prepared on ice for 30 min with immunoprecipitation lysis buffer. Supernatants were then treated with protein G beads (GE Health Care) for removal of immunoglobulin G from the neutrophil lysate. For the Btk-precipitation assay, purified Btk recombinant proteins or control recombinant protein were eluted and proteins were measured by BCA protein assay (Pierce). Bacterial supernatants were bound to nickel–nitrilotriacetic acid Sepharose beads (Qiagen) and bound recombinant proteins were eluted, then equimolar amounts of recombinant proteins were rebound to the nickel beads; afterward, samples were washed and then incubated overnight at 4 °C with the cell lysates. Beads were washed four times with lysis buffer and assessed by immunoblot analysis with anti-Mal. Before incubation with cell lysates, the amount of the recombinant protein rebound to nickel beads was assessed by immunoblot analysis with anti-histidine, and the 'dose' was readjusted for further precipitation assays.

Statistical analysis. Student's *t*-test was used for statistical analysis. The software GraphPad Prism 4 was used for these analyses.

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