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33. RNAs were prepared as described (*32*) and reverse transcribed into cDNAs with oligo(dT) as primer. For PCR analysis, the following pairs of oligonucleotides were used: globin, 5′-CACAACCCCAGAAACAGACA-3′ (sense amplimer) and 5′-CTGACAGATGCTCTCTTGGG-3′ (antisense amplimer), generating a fragment of 528 bp; actin, 5′-GGCGGACTGTTACTGAGCTGCG-3′ (sense amplimer) and 5′-AGAAGCAATGCTGTCACCTTCCCC-3′ (antisense amplimer), generating a fragment of 455 bp. PCR conditions were 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min for globin; and 35 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 0.5 min for actin. The PCR products were separated on 1.5% agarose gels, and the PCR fragments were positively identified by Southern (DNA) blotting with internal oligonucleotides.
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36. Cells were labeled with 5-hydroxy[G-^{3}H]tryptamine creatinine (^{3}H-serotonin) (Amersham, Buckinghamshire, U.K.) at 1 μCi/ml for 8 hours, washed, and incubated with monoclonal mouse anti–DNP-IgE (SPE-7; Sigma, St. Louis, MO) (10 μg/ml) or medium alone for 1 hour on ice. Cells were subsequently stimulated for 10 min at 37°C with medium alone, with monoclonal rat antibody to mouse IgE (R35-72; PharMingen, San Diego, CA) (2 μg/ml) or DNP–human serum albumin (HSA) (1 μg/ml; Sigma), or with ionomycin (1 μM; Sigma).
37. *W/W^v* recipient mice were F_1 animals derived from crosses of C57BL/6-*W^v*/+ × WB-*W*/+ parents (Japan-SLC, Japan).
38. We thank K. Kretzschmar for expert technical assistance; E. Schmidt (Mainz) for advice on mast cell staining; S.-I. Nishikawa (Kyoto University, Kyoto) for providing ACK-4 monoclonal antibody to c-Kit; E. Wagner and the Animal Facility staff (Basel) for their care in maintenance of mouse colonies; S. Meyer for expert cell sorter operation; P. Fox for technical assistance with the electron microscopy; H. Stahlberger for artwork; B. Pfeiffer and H. Spalinger for photography; and P. Ghia, K. Karjalainen, F. Melchers, and S. Takeda (Basel) and C. Lantz and M. Tsai (Boston) for discussions and critical reading of the manuscript. The Basel Institute for Immunology was founded and is supported by F. Hoffmann–La Roche, Basel, Switzerland. Supported by NIH grants AI-33372 (A.M.D.) and CA/AI-72074 and AI/CA-23990 (S.J.G.).

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Activation of BTK by a Phosphorylation Mechanism Initiated by SRC Family Kinases

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Bruton's tyrosine kinase (BTK) is pivotal in B cell activation and development through its participation in the signaling pathways of multiple hematopoietic receptors. The mechanisms controlling BTK activation were studied here by examination of the biochemical consequences of an interaction between BTK and SRC family kinases. This interaction of BTK with SRC kinases transphosphorylated BTK on tyrosine at residue 551, which led to BTK activation. BTK then autophosphorylated at a second site. The same two sites were phosphorylated upon B cell antigen receptor cross-linking. The activated BTK was predominantly membrane-associated, which suggests that BTK integrates distinct receptor signals resulting in SRC kinase activation and BTK membrane targeting.

BTK is a member of the BTK/TEC family of nonreceptor tyrosine kinases (NRTKs) (*1*–*3*). These proteins are distinct among NRTKs in containing conserved NH_2-terminal regions consisting of a pleckstrin homology (PH) domain and a proline-rich sequence, in addition to their conserved SRC homology 2 (SH2), SH3, and kinase domains. Deficient function of BTK is responsible for both human X-linked agammaglobulinemia (XLA) and murine X-linked B cell immunodeficiency (XID) (*1*, *4*–*6*). The sequelae of deficient BTK function suggest that a BTK-dependent signal is required for the expansion, functional maturation, or both of B cell progenitors (pro-B) (*7*). A diverse group of receptors is capable of activating BTK (*8*–*10*). Knowledge of how these receptors control BTK activity is necessary for understanding BTK-dependent signaling and how specific BTK mutations can interrupt these events.

Several lines of evidence implicate SRC family kinases in receptor-mediated activation of BTK, including activation kinetics (*8*), studies with LYN$^{-/-}$ mice (*11*), and in vitro interaction studies (*12*). In an effort to synthesize these data, we studied the consequences of coexpression of BTK and LYN. We used vaccinia-driven expression of BTK, LYN, or both in a B cell line transformed with Epstein-Barr virus (EBV) and deficient in BTK mRNA and protein production (Fig. 1A). Coexpression of LYN and BTK increased the tyrosine phosphorylation of BTK five times relative to that present after expression of BTK alone (Fig. 1A). These results were comparable to the 5 to 10 times increase in BTK tyrosine phosphorylation that follows cross-linking of the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on mast cells, membrane IgM on B cells, and the interleukin-5 (IL-5) receptor on pro-B cells (*8*, *9*). To eliminate the potential contribution of other B cell–specific signaling pathways, endogenous LYN, or regulation through hematopoietic phosphatases, we coexpressed BTK and LYN in a nonhematopoietic cell line. Coexpression increased both tyrosine phosphorylation of BTK and BTK enzymatic activity 5 to 10 times in multiple experiments (Fig. 1B), a significantly greater magnitude than that seen after receptor-mediated activation of BTK (*8*, *9*). Coexpression of the SRC family kinase, FYN, with BTK resulted in similar increases in both BTK tyrosine phosphorylation and enzymatic activity (*13*). In contrast, coexpression of the SYK tyrosine kinase and BTK resulted in no significant increase in BTK tyrosine phosphorylation or activity (*13*, *14*). Coexpression of BTK and LYN therefore reproduces the changes in BTK tyrosine phosphorylation and activation that follow hematopoietic receptor stimulation and provides a simplified system for the study of BTK activation.

We used this system to determine whether the tyrosine kinase activities of LYN or BTK were necessary for the LYN-dependent phosphorylation of BTK. Coex-

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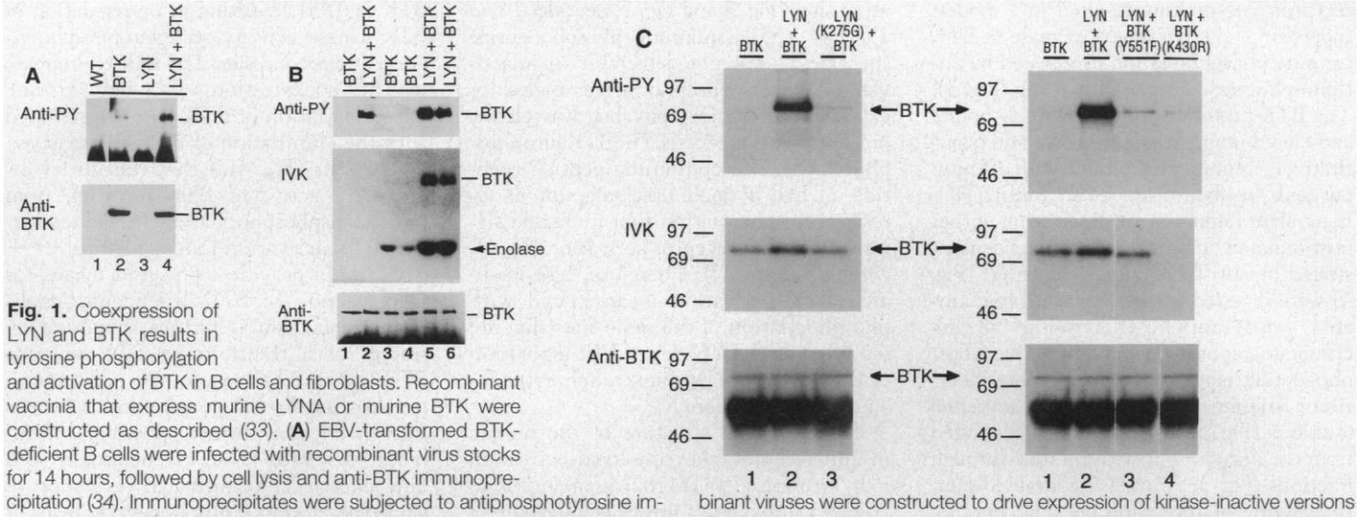

Fig. 1. Coexpression of LYN and BTK results in tyrosine phosphorylation and activation of BTK in B cells and fibroblasts. Recombinant vaccinia that express murine LYNA or murine BTK were constructed as described (*33*). (**A**) EBV-transformed BTK-deficient B cells were infected with recombinant virus stocks for 14 hours, followed by cell lysis and anti-BTK immunoprecipitation (*34*). Immunoprecipitates were subjected to antiphosphotyrosine immunoblotting (anti-PY; top) or anti-BTK immunoblotting (anti-BTK; bottom) (*35*). Lane 1, wild-type vaccinia; lane 2, BTK expression; lane 3, LYN expression; lane 4, coexpression. (**B**) Recombinant virus stocks were used to infect 3T3E cells for 8 hours (*36*). Replicate BTK immunoprecipitates were split and subjected to antiphosphotyrosine immunoblotting (top, lanes 1 to 6) or in vitro autophosphorylation-transphosphorylation assay and autoradiography (middle, lanes 3 to 6) followed by anti-BTK immunoblotting (bottom) (*37*). The positions of BTK and the exogenous substrate enolase are indicated. LYN expression was confirmed by immunoblotting of cell lysates (*38*). (**C**) Coexpression-induced tyrosine phosphorylation of BTK requires the kinase activity of both LYN and BTK. Recombinant viruses were constructed to drive expression of kinase-inactive versions of LYNA [LYN(K275G) (*39*)] and BTK [BTK(K430R)] and an activation loop, SRC-like phosphorylation site mutant of BTK [BTK(Y551F)]. 3T3E cells were infected with the indicated viruses (top) for 6 hours, followed by cell lysis, and anti-BTK immunoprecipitates were split and subjected to antiphosphotyrosine immunoblotting (upper) or in vitro autophosphorylation assay (middle) followed by anti-BTK immunoblotting (lower; dominant band is Ig heavy chain). The positions of the molecular size marker (in kilodaltons) and of BTK are indicated. Anti-LYN immunoprecipitates were also subjected to an in vitro autophosphorylation assay, followed by anti-LYN immunoblotting, which demonstrated protein expression and lack of autokinase activity in LYN(K275G) (*38*).

pression of kinase-inactive LYN(K275G) (*15*) with wild-type BTK resulted in no significant increase in BTK tyrosine phosphorylation or in in vitro kinase activity (Fig. 1C). Conversely, the LYN-dependent phosphorylation of kinase-inactive BTK(K430R) was reduced more than 90% relative to that of wild-type BTK after LYN coexpression (Fig. 1C). Autophosphorylation of a conserved tyrosine in the catalytic domains of SRC kinases (Y416) and receptor tyrosine kinases such as the insulin receptor (Y1162) is associated with an increase in their enzymatic activity (*16*–*19*). BTK contains a highly conserved SRC-like phosphorylation site within its kinase domain (Y551). Mutation of this site in BTK(Y551F) reduced by more than 90% the coexpression-associated increase in both BTK tyrosine phosphorylation and the BTK autokinase activity (Fig. 1C). The low LYN-dependent phosphorylation of BTK(K430R) and of BTK(Y551F) alleviates the concern that LYN overexpression results in indiscriminate LYN-dependent phosphorylation. Thus, maximal LYN-dependent BTK tyrosine phosphorylation requires the kinase activities of both LYN and BTK, as well as an intact SRC-like phosphorylation site at Y551, which suggests that activation of BTK requires both LYN-dependent BTK transphosphorylation and BTK autophosphorylation.

To determine whether interaction between any single protein interaction domain of BTK and LYN was required for LYN-dependent tyrosine phosphorylation of BTK, we compared proteins containing mutations of the BTK proline-rich region, PH, SH2, and SH3 domains with wild-type BTK for the ability to become tyrosine-phosphorylated. Neither these mutations nor elimination of the LYN SH2 or SH3 domains altered the coexpression-dependent activation of BTK (*13*). Thus, in the presence of high coexpression, the catalytic specificity of LYN was sufficient for tyrosine phosphorylation and activation of BTK and was not significantly affected by inactivation of any single protein domain.

The requirement of the kinase activities of both LYN and BTK for the coexpression-induced increase in BTK tyrosine phosphorylation and activation suggested that these changes resulted from at least two independent phosphorylation events. We therefore performed phosphopeptide mapping of in vivo ^{32}P-labeled BTK expressed alone and coexpressed with LYN (Fig. 2). Expression of BTK alone resulted in a relatively simple phosphopeptide pattern consisting of two predominant tryptic peptides (Fig. 2A). Phosphoamino acid analysis of these peptides revealed that they contained phosphoserine (*20*). In contrast, coexpression of LYN and BTK resulted in the appearance of two additional major phosphopeptide fragments, peptides 1 and 2 (Fig. 2B). Phosphoamino acid analysis of these peptides revealed only phosphotyrosine (*20*). Because of the reduction in the coexpression-associated increase in BTK tyrosine phosphorylation by mutation of either BTK(Y551F) or BTK(K430R), phosphopeptide mapping was done to determine the effects of these mutations on the tyrosine-phosphorylated peptides 1 and 2. The BTK(Y551F) mutation completely abolished the LYN-associated tyrosine phosphorylation of peptide 1 while retaining peptide 2 (Fig. 2C). This result suggests that peptide 1 contains Y551. In contrast, BTK(K430R) coexpressed with LYN resulted in preservation of phosphorylation of peptide 1, but loss of phosphorylation of peptide 2 (Fig. 2D), showing the presence of a BTK autophosphorylation site in peptide 2. Preliminary studies with the coexpression of LYN and mutant BTK proteins have mapped this autophosphorylation site within the SH3 domain of BTK (*21*). The LYN-dependent phosphorylation of BTK therefore consists mainly of two events: a LYN-dependent phosphorylation of the SRC-like phosphorylation site at Y551 (on peptide 1) and a BTK autophosphorylation of another tyrosine residue (on peptide 2).

We next sought to determine if this transphosphorylation-autophosphorylation mechanism was consistent with BTK activation mediated through membrane IgM stimulation of B cells. Ramos B cells negative for EBV were phosphate-labeled in vivo and activated by IgM cross-linking (Fig. 3). Consistent with previous studies,

receptor cross-linking resulted in a modest (approximately five times) increase in BTK tyrosine phosphorylation as assessed by antiphosphotyrosine immunoblotting (Fig. 3). The BTK protein was quantitatively bound and eluted from phosphocellulose and quantitatively immunoprecipitated with affinity-purified antibody to BTK (anti-BTK). Equivalent amounts of BTK from either unstimulated or stimulated cells (as demonstrated by anti-BTK immunoblotting) (Fig. 3) were digested with trypsin and fractionated with antiphosphotyrosine affinity chromatography to generate in vivo phosphopeptide maps from the antiphosphotyrosine column flow-through and enriched fractions (Fig. 3, A to D). Maps generated from the antiphosphotyrosine flow-through fractions (Fig. 3, A and C) contained multiple serine or threonine phosphopeptides, including one prominent phosphothreonine peptide that did not change after IgM ligation. Comparison of maps of the phosphotyrosine-enriched peptides before (Fig. 3B) and after (Fig. 3D) IgM stimulation revealed enhanced tyrosine phosphorylation of two peptides that migrated to positions identical to those shown to contain the BTK(Y551) and BTK autophosphorylation sites (Fig. 3 and Fig. 2, peptides 1 and 2). The Y551-containing phosphopeptide (peptide 1) was not detectable in unactivated B cells by phosphorimager analysis or extended autoradiography but was clearly present in activated cells. The BTK autophosphorylation site containing peptide (peptide 2) had a small basal amount of tyrosine phosphorylation that increased significantly after receptor activation (Fig. 3, compare B and D). Therefore, IgM-mediated BTK activation is associated with phosphorylation of the same sites that are modified after LYN-dependent activation of BTK mediated by transphosphorylation-autophosphorylation.

In the crystal structure of the human insulin receptor, the conserved activation loop tyrosine (*19*) [Y1162; homologous to BTK(Y551)] extends into the active site of the kinase domain and inhibits enzymatic activity. Transphosphorylation of this tyrosine is predicted to result in stabilization of the activation loop in a noninhibitory conformation that permits subsequent intermolecular transphosphorylation. Our results suggest that activation of BTK occurs through a similar transactivation mechanism, whereby LYN transphosphorylates BTK at Y551, resulting in up-regulation of BTK kinase activity and autophosphorylation at a second site. The limited increase in the coexpression-associated tyrosine phosphorylation of BTK that is associated with the elimination of BTK kinase activity suggests (Fig. 1C) that the initial increase in activated BTK resulting from LYN transphosphorylation facilitates further BTK activation independent of LYN. This would provide a powerful means for amplification of BTK-dependent signals. This mechanism is further supported by studies of a transforming BTK mutant, BTK* (*22*). BTK* contains a point mutation within the PH domain and is constitutively tyrosine phosphorylated and membrane associated to an extent usually seen only after receptor-mediated activation of wild-type protein. Phosphopeptide maps of BTK* contain two major tyrosine phosphopeptides that migrate in positions corresponding to the Y551 and BTK autophosphorylation site peptides identified here (*21*). The convergence of results from three different systems of BTK activation strongly supports the biological relevance of this phosphorylation sequence in BTK activation and the downstream propagation of BTK-dependent signals.

Our data are in partial conflict with a recent report that suggests that SRC kinases induce BTK activation and that the Y551F mutation can abrogate BTK autophosphorylation (*23*). We have consistently found that the BTK(Y551F) mutant has in vitro autokinase activity equivalent to the basal activity of wild-type BTK (Fig. 1C) and that only differences in experimental methodology can account for the reported lack of kinase activity in this mutant. The BTK autophosphorylation site we have identified has recently been clearly mapped (*21*), and the Y551 and autophosphorylation site containing peptides would not have been resolved with the one-dimensional cyanogen bromide mapping technique used in the previous study.

With BTK-LYN coexpression, more than 90% of tyrosine-phosphorylated BTK is present within the membrane fraction (*13*), which suggests that membrane association of BTK places it in proximity to LYN, thereby promoting LYN-dependent phosphorylation. Similarly, the PH domain point mutation in BTK* results in an increase in BTK membrane targeting and may function in part by enhancing access of BTK* to SRC family kinases (*22*). The amount of membrane-associated BTK is therefore likely to be important in regulating the strength of the BTK signal in hematopoietic cells. The BTK PH domain can bind to βγ subunits of heterotrimeric G proteins (*24*, *25*), membrane lipids (*26*), phosphokinase C (PKC) (*27*), and phos-

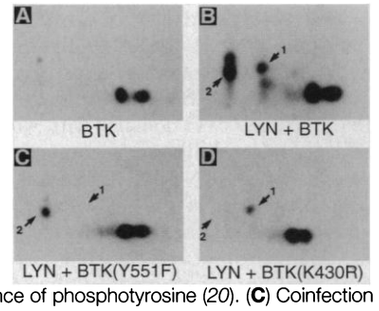

Fig. 2. Phosphopeptide analysis of the coexpression-induced phosphorylation of BTK. 3T3 cells were infected with the indicated viruses for 3 hours, washed twice in phosphate-free media, and labeled with 1 mCi of [^{32}P]orthophosphate for 3 hours in phosphate-free media. Cell lysates were immunoprecipitated with anti-BTK, and gel-isolated BTK protein was digested with trypsin and peptides separated by two-dimensional thin-layer electrophoresis and visualized by autoradiography (*32*). (**A**) Infection with BTK. (**B**) Coinfection of LYN and BTK. Arrows indicate the positions of the two major phosphopeptide fragments, peptides 1 and 2. Phosphoamino acid analysis of each of these peptides revealed the presence of phosphotyrosine (*20*). (**C**) Coinfection with LYN and BTK(Y551F) viruses, demonstrating loss of phosphorylation of peptide 1. (**D**) Coinfection with LYN and BTK(K430R), demonstrating loss of phosphorylation of peptide 2. The identities of peptides 1 and 2 were confirmed by phosphopeptide analysis of mixtures of the peptides generated in (B) with those shown in (C) or (D), respectively (*13*). Peptides were spotted in the lower left of each thin-layer chromatography plate.

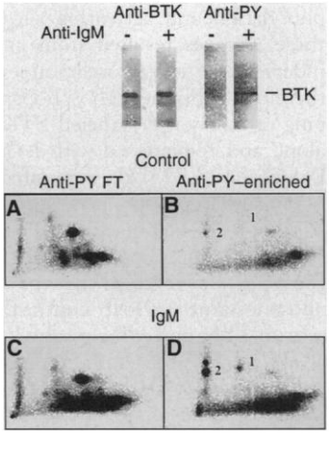

Fig. 3. Phosphopeptide analysis of BTK in B cells after receptor activation (*40*). (Top) BTK immunoprecipitates from unstimulated (−) and stimulated (+) B cells were subjected to anti-BTK immunoblotting, demonstrating equivalent recovery of BTK protein, and to antiphosphotyrosine (anti-PY) immunoblotting, demonstrating an increase in tyrosine-phosphorylated BTK after IgM cross-linking. (Bottom) Phosphopeptide maps with phosphorimager analysis of (**A**) antiphosphotyrosine flow-through (anti-PY FT) peptides from unstimulated (control) cells; (**B**) antiphosphotyrosine-enriched (anti-PY–enriched) peptides from unstimulated cells; (**C**) antiphosphotyrosine flow-through peptides from stimulated (anti-IgM$^+$) B cells; and (**D**) antiphosphotyrosine-enriched peptides from similarly stimulated B cells. Numbers indicate the positions of the two major phosphopeptide fragments, peptides 1 and 2, migrating in positions identical to those containing the BTK(Y551) site and the BTK autophosphorylation site, respectively. Peptides were spotted in the lower left of each cellulose plate.

phatidylinositol-3 kinase (*28*). These interactions or those directed by other BTK protein domains may allow BTK to integrate membrane-targeting signals with those generated by SRC kinase–linked receptor activation. Alterations of these targeting events may be involved in deficient B cell costimulatory signals in XID mice. Finally, the less dramatic increase in coexpression or phosphorylation of BTK in B cells induced by surface IgM cross-linking (Figs. 1A and 3) also suggests that negative regulation of BTK activity by PKC (*27*), or by tyrosine phosphatases such as hematopoietic cell phosphatase (HCP) (*29*), may further control BTK activation in hematopoietic cells.

Our findings predict that mutations that eliminate the ability of BTK to interact with SRC family kinases either directly or by altering the local conformation of these two major tyrosine phosphorylation sites would also result in XLA. In support of these predictions, mutations within the activation loop of BTK (R562W and R562P) (*15*) have been identified in two patients with XLA (*30*). Understanding the mechanisms controlling BTK activation may have therapeutic implications in the control of B cell malignancy and autoimmunity.

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33. Recombinant vaccinia expressing murine LYNA were constituted as described (*14*). Wild-type and mutant murine BTK complementary DNA constructs generated by site-directed or polymerase chain reaction (PCR) mutagenesis (*1, 22*) were subcloned into the pSC-65 vaccinia recombination plasmid. BTK recombinant viruses and a virus with the pSC-65 plasmid alone (control) were selected with standard techniques [P. L. Earl, N. Cooper, B. Moss, *Current Protocols in Molecular Biology* (Green and Wiley-Interscience, New York, 1987)].
34. The EBV-transformed XLA B cell line MB (D. J. Rawlings and O. N. Witte, unpublished data), which was BTK-deficient, was infected in RPMI with 10% calf serum with 8 plaque-forming units (PFU) per cell of the indicated viruses (Fig. 1A) for 14 hours.
35. BTK immunoprecipitations and protein immunoblotting were performed as described (*1*) [D. C. Saffran *et al.*, *N. Engl. J. Med.* **330**, 1488 (1994)] with an affinity-purified BTK antiserum. The monoclonal phosphotyrosine antibody 4G10 or 4G10-Biotin (1.0 μg/ml; Upstate) and horseradish peroxidase (HRP)–conjugated sheep secondary antibody to mouse or strepavidin-HRP conjugate (Amersham) were used, respectively, for antiphosphotyrosine immunoblots as recommended by each manufacturer.
36. 3T3E cells are NIH 3T3 fibroblasts that have been transfected with the α, β, and γ subunits of the rat high-affinity IgE receptor and have been previously described (*14*). Infections were performed by seeding approximately 4×10^6 to 5×10^6 3T3E cells into 150-cm^2 flasks 18 hours before infection. The monolayer was washed with Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin and 25 mM Hepes, and then overlaid with 20 ml of the same medium containing 5 to 7 PFU per cell of the indicated viruses (Fig. 1B). Single infections contained an additional 5 to 7 PFU per cell of control virus. Cells were placed at 4°C for 30 min to synchronize the infection and then grown for 6 hours at 37°C. Cells were lysed in 150 mM NaCl, 200 mM sodium borate (pH 8.0), 5 mM EDTA, 5 mM NaF, 5 μM leupeptin, 10 μM pepstatin, 10 μM aprotinin, and 1 mM sodium vanadate. Lysates were spun at 14,000 rpm for 20 to 30 min in an Eppendorf microfuge and the supernatant was subjected to the indicated immunoprecipitations. Coexpression of BTK and LYN in NIH 3T3 cells produced results identical to those obtained with 3T3E cells (*13*).
37. In vitro kinase and enolase transphosphorylation assays were performed as described (*1*) with the following modifications: immunoprecipitates were washed in kinase buffer consisting of 150 mM NaCl, 30 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 100 μM sodium vanadate and incubated for 5 min at room temperature in a reaction mix containing 10 μM adenosine triphosphate (ATP), 10 μg of acid-denatured enolase, and 10 μCi of [γ-^{32}P]ATP, boiled in sample buffer, separated with 10% SDS-PAGE, and subjected to antiphosphotyrosine immunoblotting and autoradiography.
38. The antibody to LYN, immunoprecipitation, in vitro kinase assay, and blotting protocols were as described (*13, 14*).
39. We constructed the LYN(K275G) mutant by using two-step overlap PCR to change bases 908 and 909 of the wild-type murine LYN cDNA from AA to GG, resulting in substitution of Gly for Lys at position 275. The presence of the desired mutation and the absence of other mutations was confirmed by dideoxy chain termination sequencing.
40. Cells (4×10^8) were washed twice in phosphate-free RPMI and labeled with 40 mCi of [^{32}P]orthophosphate for 5 hours in 8 ml of phosphate-free RPMI containing 0.1% dialyzed fetal calf serum. Sodium orthovanadate (100 mM final concentration) was added for the final hour of labeling. One-half of the labeled cells were activated with goat antibody to human IgM (200 μg of total antibody; Southern Biotech) for 10 min before cell lysis in ×1 Triton lysis buffer [3% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM tris (pH 7.4), 2 mM EDTA, 10 mg/ml each of aprotinin and leupeptin, 0.5 mM phenylmethylsufonyl fluoride, 10 mM sodium fluoride, and 1 mM sodium orthovanadate]. BTK in cell lysates was enriched with phosphocellulose (PC) chromatography (8×10^7 cpm recovered from each, unstimulated and stimulated) and immunoprecipitated with affinity-purified BTK antibody (recovery: 2×10^4 and 4×10^4 cpm, unstimulated and stimulated, respectively). Equal amounts of BTK from unactivated and activated cells were digested on nitrocellulose with trypsin; tyrosine-phosphorylated peptides were enriched with antiphosphotyrosine affinity chromatography and subsequently separated by two-dimensional thin-layer electrophoresis and visualized by autoradiography (*32*).
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