TFII-I is a ubiquitously expressed multifunctional transcription factor with broad biological roles in transcription and signal transduction in a variety of cell types. We and others have shown that TFII-I can interact physically and functionally with Bruton's tyrosine kinase (Btk), a hematopoietic non-receptor protein tyrosine kinase that is critical for B lymphocyte development. Although TFII-I-Btk interactions are impaired in B cells from X-linked immunodeficient mice, the precise molecular determinants governing TFII-I-Btk complex formation remain unknown. To this end, we have conducted a structural analysis of TFII-I-Btk interactions by using a panel of TFII-I mutants. These studies have revealed that a region within the N-terminal 90 amino acids of TFII-I, which includes a putative leucine zipper motif, primarily responsible for its interaction with Btk. Mutations in the leucine zipper region itself were not sufficient to abrogate binding of TFII-I to Btk, suggesting that regions/residues outside the leucine zipper are responsible for such interactions. Because the first 90 amino acids of TFII-I are required for its dimerization, we propose that Btk tethers TFII-I to the cytoplasm by preventing its dimerization and its subsequent nuclear localization. We further examined the requirement of tyrosine phosphorylation for TFII-I-Btk complex formation. Our data showed that Src-dependent tyrosine phosphorylation sites in TFII-I are not targeted by Btk, suggesting that multiple kinases can independently target TFII-I via distinct signaling pathways. Our results provide a beginning step toward understanding the functional importance of the TFII-I-Btk pathway in B cell signaling and gene expression.

Signaling through the surface antigen receptor in B cells leads to the activation of a variety of non-receptor protein tyrosine kinases (1). One of these key enzymes is Bruton’s tyrosine kinase (Btk). Various mutations in Btk have been identified and are known to cause X-linked agammaglobulinemia in humans (2, 3) and X-linked immunodeficiency (Xid) in mice (4, 5). Although the number of mature B cells is virtually absent in X-linked agammaglobulinemia, Xid is characterized by a less severe phenotype in which mature B cells capable of responding to antigenic stimulation are severely diminished (4). In addition, genetic depletion of Btk in mice also results in an Xid phenotype whereby pre-B to mature B cell differentiation is drastically hampered, further underscoring the importance of Btk in B cell function (6, 7). Despite these observations, the downstream targets of Btk are largely unknown. Results from our laboratory and the results of others (8–10) have shown that Btk associates physically and functionally with the transcription factor TFII-I, thereby providing a mechanism for one of the pathways that connects Btk-mediated signaling to gene activation. Given that Btk is predominantly a cytoplasmic kinase and TFII-I is a nuclear transcription factor, it is important to decipher how they assemble. We present here a detailed mechanistic study, which specifically addresses this question.

TFII-I is a multifunctional transcription factor that is induced by a variety of external signals (8–12). In normal resting splenic B cells, TFII-I is largely found in the cytoplasm, a significant portion of which is tethered to Btk. Upon cross-linking of the surface B cell receptor, TFII-I is tyrosine-phosphorylated in a Btk-dependent fashion and translocates from the cytoplasm to the nucleus (8). In immunocompromised Xid B cells, this process is deregulated such that TFII-I is constitutively localized in the nucleus (8). In separate studies, we have also shown that in murine fibroblasts (which naturally lack Btk), TFII-I is regulated by c-Src (13). The major Src-dependent tyrosine phosphorylation sites have been described and are shown to be important for growth factor-mediated transcriptional activity of TFII-I (13). These studies have collectively raised two major questions. First, by what mechanism does Btk retain TFII-I in the cytoplasm? Second, are the regulatory pathways by which Btk and Src activate TFII-I mutually exclusive? Alternatively, it is possible that targeting of TFII-I by Btk and Src at different phosphorylation sites might imply that TFII-I can be regulated via multiple and independent pathways. In order to answer some of these questions, we employed a battery of TFII-I mutants and analyzed their potential to interact physically and functionally with Btk. First, our results indicate that the association of TFII-I with Btk occurs primarily via the N-terminal 90 amino acids of TFII-I, which include a putative leucine zipper motif (14). Contrary to our expectations, mutations disrupting the zipper did not abolish interactions significantly, suggesting that residues/motifs outside of the zipper region are primarily contributing to these interactions. More significantly, the same 90 amino acids of TFII-I are also required for its dimerization. These data support a model in which Btk is able to retain TFII-I in the cytoplasm by preventing its dimerization. Second, we found that mutations in Src-dependent tyrosine phosphorylation sites in TFII-I neither disrupted their physical interactions with Btk nor did they have any appreciable effect on Btk-dependent transcriptional...
activation of a TFII-I-responsive promoter. Therefore, we propose that Btk and c-Src can target TFII-I through different pathways and via different tyrosine residues, suggesting that distinct and independent signaling pathways can simultaneously regulate TFII-I.

EXPERIMENTAL PROCEDURES

Cell Culture

Cos-7 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (Atlantic Biologicals), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Cells were grown at 37 °C, in 5% CO2, and subcultured two or three times weekly.

Plasmids

TFII-I Plasmids—The construction of the GST fusion plasmids pEBG vector, pEBG-II-I wild type, pEBG-II-I-YY248/249FF, pEBG-II-IYY248/249FF-Y611F, pEBG-II-I-ΔDR, pEBG-II-IΔNSL1, pEBG-II-I-ΔN90, pEBG-II-I-p70, pEBG-II-I-p46, pEBG-II-I-R1, pEBG-II-I-R2, pEBG-II-J8, and pEBG-II-Jo has been detailed elsewhere (14, 15). The construction of the GFP fusion plasmids pEBB-GFP-II-ΔΔ, pEBB-GFP-II-J8, and pEBB-GFP-II-Jo has been detailed elsewhere (15).

PCR-mediated mutagenesis was used to generate the tyrosine phosphorylation mutants pEBG-II-I-Y277F and pEBG-II-I-YY248/249FF+Y277F and the structural mutants pEBG-II-I-ΔPPI, a polyproline region PPII helix deletion mutant (amino acids 291–298), pEBG-II-I-ΔN90 (amino acids 1–90), and the leucine zipper mutants pEBG-II-I-ΔL30p, pEBG-II-I-L30p, and pEBG-II-I-ΔL30p+L37p.

pEBG-II-I-Y277F Mutant—The primers used to generate the Y277F mutant are as follows: PCR 1, primer 1 (5′-ACAGTGGTACCTGTAC-3′) and primer 2 (5′-AAATATGATCTTTTCTACCATCTTTG-3′), PCR 2, primer 3 (5′-GAGGATGTTTCTCTCTCAAG-3′) and primer 4 (5′-GGTTGTACGTAGCATGTAGTG-3′) and pEBG-II-I-GFP was used as template for these reactions. Primers 1 and 4 were used to carry out a third PCR by using reactions 1 and 2 as templates. The final product was gel-purified, digested with Acc65I and SnaB1, and subsequently ligated into pEBG-II-I, digested with the same restriction enzymes. pEBG-II-I-YY248/249FF+Y277F Mutant—The primers used to generate the YY248/249FF+Y277F mutant were the same as for Y277F, except that pEBG-II-YY248/249FF was used as template in reactions 1 and 2. Primers 1 and 4 were used to carry out a third PCR by using reactions 1 and 2 as templates. The final product was gel-purified, digested with BamHI and KpnI, and subsequently ligated into pEBG-II-I, digested with the same restriction enzymes.

pEBG-II-I-L37P Mutant—The primers used to generate the L30P+L37P mutant were the same as for L37P, except that pEBG-II-I-ΔL30p was used as template in reactions 1 and 2. Primers 1 and 4 (described for pEBG-II-I-ΔL30p as well as for pEBG-II-I-ΔL30p+L37p) were used to carry out a third PCR by using monoglobal reaction 1 and 2 as templates. The final product was gel-purified, digested with BamHI and KpnI, and subsequently ligated into pEBG-II-I, digested with the same restriction enzymes.

Btk Plasmids—The hemagglutinin-tagged wild type pGlo-BTK-HA and mutant pGlo-K430E-HA constructs were a kind gift of Genhong Chen, and have been described previously (18). The plasmid pDNAs.1+ /Zeo-FLAG-Btk was a kind gift from Dr. Andrew Chan (Genentech).

Other Plasmids—The expression plasmid encoding wild type RC-CMV-c-Src has been described previously (16). The c-fos-luciferase reporter plasmid (pSVOA5+) containing a 379-bp murine c-fos promoter upstream of a luciferase reporter gene, and the pRL-TK (thymidine kinase) (Renilla luciferase) plasmid (Promega) have both been described previously (15).

Transient Transfection of COS-7 Cells and Luciferase Reporter Assays

2 × 105 COS-7 cells were seeded in 6-well plates. 24 h later, cells were transfected using supplemented DMEM with Polyfect lipofection reagent according to the manufacturer’s recommendations (Qiagen). 600 ng of c-fos-luciferase promoter-luciferase plasmid were combined with 35 ng of Renilla luciferase pRL-TK (internal control). Varying concentrations of pEBG-II-I wild type and pEBG-II-I phosphorylation mutants were added to the mixture in the presence or absence of pGlo-BTK-HA. Total DNA concentration was normalized using empty pEBG vector. DNA-Polyfect mixtures were added to the cells 24 h post-transfection, fresh supplemented DMEM was added to the cells. Cells were harvested 40 h post-transfection according to manufacturer’s instructions, using passaging lysis (Promega). Relative luciferase activities of firefly and Renilla luminescence were measured using the Dual Luciferase Assay kit (Promega). Transfections were performed in triplicate and reporter assay experiments repeated more than three times.

Cell Extracts, GST Pull-down and Immunoprecipitation Assays, Western Blot Analysis

Cos-7 cells were transfected at 80% confluency in 10-cm tissue culture plates using Polyfect lipofection reagent according to manufacturer’s instructions (Qiagen). 8 μg of pEBG-II-I wild type and mutant variants were transfected in the absence or presence of either pGlo-BTK-HA (20 μg) or pDNAs.1+ /Zeo-FLAG-BTK (30 μg). DNA-Polyfect mixtures were added to the cells 24 h post-transfection, fresh supplemented DMEM was added to the cells. Cells were harvested 40 h post-transfection, rinsed twice with phosphate-buffered saline (PBS), and lysed for 30 min at 4 °C (rotating motion) in lysis buffer (25 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM NaF, 2 mM Na3VO4, 1 mM EDTA, 1% Triton X-100, and 1% Nonidet P-40), supplemented with EDTA-free 1× anti-protease mixture (Roche Applied Science). Lysates were pre-cleared by centrifugation 15 min, 14,000 rpm, at 4 °C. Total protein concentration was measured using the Bradford method (Bio-Rad). 500–1000 μg of whole cell lysates were used for GST pull-down assays, and 800–1000 μg were used for immunoprecipitations with either anti-HA mouse monoclonal antibody (12CA5, Roche Applied Science) or anti-FLAG mouse monoclonal antibody (M2, Sigma).

For immunoprecipitations, antibodies were incubated with whole cell lysates for 20 min at 4 °C (rotating motion), followed by an incubation with protein G-Sepharose “4 Fast Flow” beads (1:1 slurry, 63 μl) (Amersham Biosciences) for 1 h 40 min at 4 °C (rotating motion). For Western blot analyses, whole cell extract, immunoprecipitation, or GST pull-down samples were resuspended in 4× SDS-PAGE Laemmli Buffer and boiled 5 min in a 100 °C heat block. Subsequently, samples were subjected to 10% SDS-PAGE. Gels were transferred to nitrocellulose using a semi-dry transfer apparatus as described (15). Blots were then blocked in 5% nonfat milk or 5% bovine serum albumin (Sigma) (the latter used for anti-phosphotyrosine immunoblotting). The following primary antibodies were used for immunoblotting: anti-HA and anti-FLAG (see above) (1:2000 dilution), anti-TFII-I (rabbit poly-
clonal) (1:2500) (11), anti-GST (GST-2 mouse monoclonal, Sigma) (1:3500), anti-GFP (JL-18 mouse monoclonal, Clontech) (1:2000), anti-Btk (rabbit polyclonal, BD Pharmingen) (1:1000), and anti-α-Src (BC-12, Santa Cruz Biotechnology) (1:2000). Secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were used at 1:10,000 dilution (Zymed Laboratories Inc.). Western blots were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences) using standard methods. Autoradiographs were image-scanned, and densitometry was performed to quantitate the intensity of Western blot bands using appropriate background correction. Individual experiments were repeated at least 3 times.

**Immunostaining Analysis by Fluorescence Microscopy**

2 x 10^6 COS-7 cells were seeded in 6-well plates over glass coverslips overnight. Cells were transfected using Polyfect lipofection reagent according to the manufacturer's recommendations (Qiagen). In brief, DNA-Polyfect mixtures were added to cultures (200 ng of GST-tagged TFII-I or mutant N90 TFII-I cDNA constructs in the absence or presence of 200 ng of FLAG-tagged wild type Btk). 24 h post-transfection, cells were gently rinsed in PBS buffer and fixed in 4% paraformaldehyde (Sigma). Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma), rinsed, and blocked in 10% fetal bovine serum (Invitrogen). Cells were immunostained with a 1:3500 dilution of anti-GST primary antibody (GST-2 mouse monoclonal, Sigma). Cells were rinsed and incubated with a 1:15,000 secondary antibody dilution of goat anti-mouse IgG conjugated with Alexa 488 fluorochrome (Molecular Probes) (green, Fig. 6). Cells were rinsed, and a sequential incubation with a second primary antibody was performed using a 1:2000 dilution of anti-HA antibody (kind gift of Dr. David Rawlings). After rinsing, cells were incubated with a 1:15,000 secondary antibody dilution of goat anti-rabbit IgG conjugated with Alexa 594 fluorochrome (Molecular Probes) (red, Fig. 6). Subsequently, cells were washed in PBS and nuclei stained in a 300 nM solution of 4',6-diamidino-2-phenylindole dihydrochloride reagent (DAPI) (Molecular Probes). Cells were subsequently rinsed and mounted on slides. Slides were examined on a Nikon Eclipse E400 fluorescence microscope and photographed using “Spot-Advanced” software. An average of 100 cells was examined in random fields at ×100 magnification. Results are representative of two independent experiments.

**RESULTS**

**Analysis of TFII-I-Btk Interactions: Structural and Phosphorylation-deficient Mutants of TFII-I**—Previous work from our laboratory and of others (8, 9) showed that TFII-I associates with Btk both physically and functionally, TFII-I subsequently becomes tyrosine-phosphorylated, and upon receptor cross-linking in B cells, translocates into the nucleus where it modulates transcriptional events (8). In order to understand the biological importance of Btk-mediated activation of TFII-I, it becomes imperative to determine what are the molecular mechanisms that drive such protein interactions. It is known that the pleckstrin homology domain of Btk is important for its binding to TFII-I (8, 9, 17). In contrast, the binding domains for Btk within TFII-I have not been mapped. Noteworthy is the fact that several spliced isoforms of TFII-I exist and that interactions of these isoforms with Btk have not been examined. Thus, to elucidate the functional domains of TFII-I that are responsible for its association with Btk, we conducted a systematic analysis of TFII-I-Btk interactions by using a panel of TFII-I mutants (expressed as GST fusion proteins). The TFII-I protein has a basic amino acid-rich DNA binding domain (BR), an activation domain at the C-terminal end, an N-terminal region containing a putative dimerization domain, six reiterated I-repeats, each containing a helix-loop-helix domain, and several tyrosine residues that are targets of growth factor-mediated signaling (13–15). To determine the role of these TFII-I domains in interaction with Btk, mutants were generated accordingly. These mutants fall into four categories. Mutants that lacked N- or C-terminal ends, viz. ΔN90, lacked a potential dimerization domain; p70 lacked the activation domain and behaved as a dominant negative, and p46 contained the N-terminal half of the protein (14). Internal mutants lacked either the DNA binding/basic region (ΔBR), the nuclear localization signal (ΔNLS1), or the putative SH3 binding domain (ΔPP1I) (13, 14). The third category of mutants were fragments of TFII-I containing isolated I-repeats R1 and R2 known to mediate interactions between isoforms of TFII-I and the N-terminal 90 amino acids of TFII-I that contain the putative dimerization domain (14). In addition, we investigated whether tyrosine phosphorylation is a requirement for TFII-I-Btk complex formation by using mutants (tyrosine to phenylalanine) of TFII-I and co-expressing them with wild type HA-tagged Btk. These mutants were ectopically co-expressed with HA-tagged or FLAG-tagged Btk in COS-7 cells. The schematization of deletion mutants (Fig. 1A) as well as phosphorylation-deficient point mutation constructs (Fig. 1B) of TFII-I is depicted in Fig. 1.

A control experiment was included in our study to corroborate previous data showing that ectopically expressed GST-tagged TFII-I interacted with HA-tagged Btk in COS-7 cells (8) (Fig. 2A). However, in the absence of ectopically expressed GST-TFII-I, the anti-HA antibody failed to precipitate any nonspecific proteins of the same molecular weight range as GST-TFII-I (146 kDa) (Fig. 2A). The blot was probed with anti-TFII-I antibody (top panel) showing that GST-TFII-I could be co-precipitated in the presence of HA-Btk. Importantly, GST-TFII-I was not immunoprecipitated with anti-HA antibody when expressed in the absence of HA-Btk (data not shown and Ref. 8). The input lane (WCE) showed the expression level of GST-TFII-I. Moreover, the same blot was stripped and re-probed with anti-HA antibody. Although the level of HA-Btk expression was low in WCE lanes, the IP lanes clearly showed that HA-Btk was immunoprecipitated.

Moreover, in order to begin delineating the contact regions between TFII-I and Btk, we investigated whether isolated I-repeats of TFII-I could by themselves associate with Btk. The I-repeats of TFII-I contain helix-loop-helix motifs, which are known to facilitate protein-protein interactions (14). We chose the R1 and R2 I-repeats of TFII-I based on previous data indicating that these isolated domains are capable of dimerizing with TFII-I (14). An immunoprecipitation assay using anti-HA antibody was conducted on whole cell lysates expressing R1 and R2 (Fig. 2B). This was followed by immunoblotting with anti-GST antibody (top panel). Whole cell extracts were included as controls for input level comparison with immunoprecipitated samples. The blot was also stripped and re-probed with anti-HA antibody to assess overall Btk protein levels (bottom panel). Our results indicate that the isolated repeats R1 and R2 failed to interact with Btk, suggesting that these individual domains were not sufficient to mediate TFII-I-Btk interactions. This experiment further showed that the anti-HA antibody failed to precipitate any GST fragment of TFII-I, indicating that nonspecific binding via the GST moiety was minimal under our conditions.

**Interaction and Tyrosine Phosphorylation of Spliced Variants of TFII-I with Btk**—There are at least four splice variants of TFII-I naturally occurring in mammals (15, 18, 19). The α-isofrom is not expressed in murine cells but is expressed in human cells; the β-isofrom is expressed in both murine and human cells; the Δ-isofrom is expressed in both human and murine cells and is the most widely studied TFII-I variant (18, 19). Because the γ-isofrom is predominantly, if not exclusively, expressed in neuronal cells (18, 19), we decided not to focus on this isofrom for our studies. We have shown previously (15) that the TFII-I-α, TFII-I-β, and TFII-I-δ isoforms are capable of forming homomeric as well as heteromeric interactions with each other. This prompted us to test whether different isoforms of TFII-I could differentially bind to Btk. Hence, TFII-I-α, TFII-
I-β, and TFII-I-Δ isoforms were expressed in COS-7 cells as GST fusion proteins in the presence of ectopic Btk (expressed as a HA fusion protein). Btk was immunoprecipitated using an anti-HA antibody, and its protein complex formation with different TFII-I isoforms was subsequently visualized using an anti-GST antibody (Fig. 2C). Whole cell extracts were included as controls for input level comparison with immunoprecipitated samples. Results show that the Δ-, α-, and β-isoforms of TFII-I can all associate with Btk to a similar extent. The immunoblot was stripped and re-probed with anti-HA antibody to assess overall Btk protein levels (bottom panel).

Subsequently, we further examined whether the three TFII-I isoforms tested displayed any differences in tyrosine phosphorylation upon interaction with Btk. Variations in tyrosine phosphorylation between isoforms upon co-expression with Btk might provide a clue as to either functional and/or domain-specific differences intrinsically exhibited by the TFII-I splice variants. Hence, tyrosine phosphorylation status of the TFII-I isoforms in the presence of Btk was analyzed via GST pull-down using an anti-phosphotyrosine (Anti-p-Tyr) antibody (Fig. 3A, top panel). The immunoblot was stripped and re-probed with anti-GST to detect comparable TFII-I expression levels (middle panel). The immunoblot was stripped a second time and re-probed with anti-HA antibody to detect comparable Btk protein levels (bottom panel). Results indicated that the α-, β-, and Δ isoforms all exhibited similar tyrosine phosphorylation levels when co-expressed with Btk (Fig. 3A).

Furthermore, in order to demonstrate that the tyrosine phosphorylation of TFII-I isoforms was due to the kinase activity of Btk and not of other ubiquitous tyrosine kinases present in COS-7 cells, a GST pull-down experiment was conducted in which tyrosine phosphorylation levels of TFII-I-Δ or TFII-I-β were assessed in the absence or presence of wild type Btk, via immunoblotting using an anti-phosphotyrosine antibody (Fig. 3B). In addition, we included in this experiment a condition where the tyrosine phosphorylation levels of TFII-I were measured in the presence of a kinase-inactive mutant form of Btk, K430E, anticipating that under this condition little or no tyrosine phosphorylation of pulled down TFII-I should be observed. Indeed, results indicated that both TFII-I-Δ and TFII-I-β exhibited heightened tyrosine phosphorylation levels when in the presence of wild type Btk, but not in its absence, or when in the presence of mutant K430E-Btk (Fig. 3B, top panel). The immunoblot was stripped and re-probed with anti-GST to monitor TFII-I pull-down levels (bottom panel). Even though the amount of GST-TFII-I precipitates in the absence of Btk was slightly lower than the amount of GST-TFII-I in the presence of wild type Btk, the normalized basal phosphorylation of TFII-I isoforms in the absence of wild type Btk was still negligible (~5-fold lower), as was the phosphorylation in the presence of K430E (~4-fold lower), further corroborating previous findings from our group and from others (8, 9). Similar results have also been observed with the α isoform (data not shown). Our data also demonstrated that tyrosine phosphorylation of TFII-I isoforms-Δ, α, and β was comparable upon co-expression with Btk, and therefore not due to isoform-specific differences (Fig. 3A).

A control experiment was performed in order to demonstrate that the kinase activity of Btk was intact under the expression conditions employed; thus, an immunoprecipitation assay was carried out on whole cell lysates expressing either wild type Btk or a kinase-inactive mutant of Btk, K430E, using an anti-HA antibody. The tyrosine phosphorylation status of wild
type Btk was compared with that of K430E by immunoblotting with anti-phosphotyrosine antibody (Fig. 3C). Although wild type Btk was readily auto-phosphorylated at tyrosine residues, the K430E mutant was incapable of autophosphorylation (top panel) (K430E migrates at a slightly higher molecular weight.) The blot was stripped and re-probed with an anti-Btk antibody to show equivalent immunoprecipitation levels of wild type Btk or K430E mutant Btk (bottom panel). Thus, data indicated that the wild type Btk used under our experimental conditions was capable of retaining bona fide kinase activity and that the K430E mutant was indeed kinase-inactive.

Noteworthy is the fact that in immunoprecipitation and GST pull-down assays performed in the present study, controls were included for single transfectants, as well as for whole cell lysates incubated with beads alone (G-Sepharose or glutathione-agarose) or with pre-immune serum as an irrelevant antibody control for immunoprecipitations. These negative controls have convinced us that samples used in the present study did not exhibit significant levels of nonspecific protein binding to beads (data not shown, but also see Ref. 8).

Based on the results that no substantial differences in Btk binding or tyrosine phosphorylation were observed between splice variants of TFII-I, and for the sake of simplicity, we focused on TFII-I-Δ in subsequent experiments. Hence, TFII-I-Δ was used to examine which domains of TFII-I were required for mediating protein interactions with Btk.

**Deletion of the N-terminal Region of TFII-I Impairs Binding to Btk**—Previous data from our laboratory has indicated that certain deletional mutants of TFII-I, namely p70, ΔN90, and ΔBR, are transcriptionally inactive (their activity has been shown to be impaired when tested on the c-fos and Vβ5.2 promoters) (14, 20). The p70 mutant lacks the C-terminal activation domain of TFII-I; ΔN90 lacks the N-terminal 90 amino acids, containing the putative leucine zipper; and ΔBR possesses a deletion within the DNA binding domain of TFII-I (residues 301–306) (14). Furthermore, we have shown that ΔN90 fails to dimerize with wild type TFII-I-Δ isoform (14). Consequently, we investigated whether ΔN90, ΔBR, p70, and also p46, a shorter version of p70 lacking repeat R4 in addition to the C-terminal activation region (comprising R5 and R6), were capable of interacting with Btk. We carried out an immunoprecipitation assay using anti-HA to test these mutants when compared with wild type TFII-I (Fig. 4, A and B). Protein complexes were visualized using anti-GST antibody (top panels). Blots were stripped and re-probed with anti-HA antibody to reveal Btk expression levels (bottom panels). Our data indicate that whereas ΔBR and p46 were capable of interacting with Btk, ΔN90 exhibited a significantly impaired interaction with Btk (Fig. 4A). These immunoprecipitation experiments were conducted several times; normalized densitometry measurements consistently showed a decrease of at least 2.9-fold in ΔN90 binding to Btk relative to wild type TFII-I. Hence, we believe we have identified an important binding domain for Btk in TFII-I, consisting of the first 90 amino acids of the N terminus of TFII-I, which includes a putative leucine zipper.

Although it appeared that the p70 mutant manifested a slightly decreased interaction with Btk compared with wild type TFII-I (Fig. 4B), after careful examination of the densitometric values for this experiment, this apparent decrease was found to be insignificant (Fig. 4B). The ability of p46 to interact...
with Btk (−1.5–2-fold higher binding to Btk than wild type TFII-I) will be addressed under the “Discussion.” Importantly, immunoprecipitation assays revealed that the ΔNLS1 mutant is also capable of interacting with Btk, and thus, Btk does not appear to interact with and tether TFII-I to the cytoplasm by directly masking its nuclear localization signal (Fig. 4C). Although the possibility exists that Btk might mask NLS2, in our hands and in the cell types tested, NLS2 appears to be non-functional (15).

We subsequently performed additional experiments to determine whether any changes in tyrosine phosphorylation of the structural mutants relative to wild type TFII-I could be observed upon co-expression with Btk, reasoning that a lack of protein interaction might be correlated to a defect in tyrosine phosphorylation of TFII-I. Namely, we wanted to determine if tyrosine phosphorylation was required in order for these protein interactions to ensue. We observed that all mutants tested were tyrosine-phosphorylated in the presence of Btk, albeit...
with an approximate 2-fold decrease in tyrosine phosphorylation levels of the ΔN90 mutant relative to wild type TFII-I. This later observation suggests that optimal Btk-dependent tyrosine phosphorylation of TFII-I may require efficient physical interaction between the two proteins (data not shown).

The Leucine Zipper Motif within the First 90 Amino Acids of the N-terminal Region of TFII-I Is Not Critical for TFII-I-Btk Complex Formation—Because we were interested in better determining the importance of the N-terminal domain of TFII-I in facilitating TFII-I-Btk interactions, we examined whether the first 90 amino acids of TFII-I were necessary and/or sufficient to mediate TFII-I-Btk complex formation. Thus, we generated a GST-N90 fusion construct and performed an immunoprecipitation assay. N90 and wild type TFII-I were co-expressed with wild type Btk in COS-7 cells and subjected to Western blotting (WB) by using anti-GST antibody. The blot was stripped and re-probed with anti-GST antibody to monitor Btk expression levels. GST-TFII-I degradation products were visualized by Western blotting using an anti-GST antibody. The blot was stripped and re-probed with anti-GST antibody to monitor GST-TFII-I pull-down levels at a 146-kDa range. C and D, the leucine zipper motif of TFII-I is not required for TFII-I-Btk interactions. GST-tagged WT TFII-I and mutants L30P, L37P, L30P/L37P, and N90 were co-expressed with FLAG-tagged Btk in COS-7 cells and subjected to a GST pull-down assay (1000 µg) followed by Western analysis (C), and a Western blot on whole cell extracts (30 µg) (D). Anti-FLAG antibody was used to detect interactions with Btk on the GST pull-down and overall Btk levels on Western blot whole cell extracts. Blots were stripped and re-probed with anti-GST to detect GST pull-down levels in GST pull-down and Western blot.

Given that the N90 domain of TFII-I contains a putative leucine zipper motif (15), we sought to examine whether the leucine zipper itself was required in facilitating TFII-I-Btk interactions at the N-terminal portion of TFII-I. Thus, we generated three targeted mutants of the leucine zipper motif of TFII-I by creating point mutations at positions Leu30-Pro and Leu37-Pro, thus disrupting the functionality of the leucine zipper region. We subsequently tested these mutants: L30P, L37P, and the double mutant L30P/L37P along with the N90 mutant in a GST pull-down assay (Fig. 5, C and D). Moreover, because all previous data had been generated using HA-tagged Btk, it was necessary to ensure that our results were not biased by the use of this particular tag. Thus, this GST pull-down assay was carried out using whole cell lysates expressing FLAG-tagged Btk in conjunction with GST-tagged wild type or mutant TFII-I constructs (Fig. 5C). Protein complexes were visualized using an anti-FLAG antibody. The blot was stripped and re-probed with anti-GST antibody to control for TFII-I protein levels. Blots were stripped and re-probed with anti-GST to detect GST pull-down levels in GST pull-down and Western blot.

with an approximate 2-fold decrease in tyrosine phosphorylation levels of the ΔN90 mutant relative to wild type TFII-I. This later observation suggests that optimal Btk-dependent tyrosine phosphorylation of TFII-I may require efficient physical interaction between the two proteins (data not shown).
antibody at the molecular weight corresponding to GST-TFII-I (146 kDa). In contrast, the interaction between GST-TFII-I and FLAG-Btk was clearly visualized by an anti-FLAG antibody. The blot was stripped and re-probed with anti-GST antibody to indicate the level of precipitated GST-TFII-I at 146 kDa. In addition, we have performed control experiments using the GST empty vector in immunoprecipitation assays as well, and we have obtained the same results (data not shown). Input levels of total protein from whole cell lysates used in the GST pull-down (Fig. 5C) were visualized on a separate blot using anti-FLAG antibody to detect Btk expression (Fig. 5D, top panel) stripped and re-probed with anti-GST antibody to detect TFII-I protein levels (Fig. 5D, bottom panel). Surprisingly, as can be seen from anti-FLAG immunoblotting of the GST pull-down, all three leucine zipper mutants were able to interact with Btk. This indicated that disruption of the leucine zipper motif did not significantly affect the ability of TFII-I to associate with Btk and therefore that regions outside of the leucine zipper region and yet comprised within the first 90 amino acids of TFII-I were probably responsible for mediating TFII-I-Btk complex formation.

Worth indicating is the fact that we conducted further immunoprecipitation assays using anti-FLAG antibody with most of the mutants tested in this study; the results confirmed for instance that the diminished interaction between Btk and ΔN90 relative to wild type was reproducible using the FLAG tag (data not shown). Hence, the use of a particular tag (HA or FLAG) did not influence our results.

**Immunolocalization of TFII-I with Btk in COS-7 Cells**

Although we have shown physical interactions of TFII-I with Btk in primary B cell cytoplasm and consequently the level of constitutive nuclear TFII-I is higher in Xid B cells (8), whether these two proteins interact in COS cell cytoplasm has not been addressed. In order to visualize the ability of TFII-I to interact with Btk in COS cell cytoplasm, GST-tagged TFII-I DNA constructs were transiently expressed in the absence or presence of FLAG-tagged Btk. Subsequently, the extent of co-localization of either wild type or mutant TFII-I with Btk was assessed by immunofluorescence microscopy, utilizing an anti-GST antibody to detect TFII-I protein, and an anti-Btk antibody to detect Btk protein. DAPI staining was used to stain cell nuclei.

Results indicated that ectopically expressed wild type TFII-I localized predominantly to the nucleus of COS-7 cells in the absence of Btk (Fig. 6A). It is important to emphasize that in transient transfection assays in COS-7 cells, ectopically expressed wild type TFII-I naturally localizes to the nuclei of this cell type, artificially simulating an “activation”-driven translocation of TFII-I into the nucleus, due to the nature of its overexpression. The **MERGE panel** denotes the juxtaposition of fluorochrome staining, indicating that TFII-I mostly localized to the DAPI-stained nuclei. Btk localized extensively to the cellular cytoplasm in the absence of other co-expressed proteins (data not shown). However, when wild type TFII-I was co-expressed with Btk, a significantly increased expression of TFII-I in the cytoplasm was observed (Fig. 6B). The increased co-localization of TFII-I with Btk can be detected by the yellow staining in the **MERGE panel**.

In addition, we tested the ability of the N90 mutant of TFII-I to interact with Btk, anticipating that this mutant would not be able to localize to the nucleus (lacking most of the TFII-I domains including the NLS signal). However, the precise subcellular localization of this mutant was unpredictable. Immunostaining experiments showed that the N90 mutant localized extensively to the cytoplasm of COS-7 cells in a diffused pattern in the absence (Fig. 6C) and in the presence of Btk (Fig. 6D). Although this result is not a demonstration of a direct N90-Btk interaction, it is nonetheless consistent with physical interactions between N90 and Btk that were shown biochemically. Thus, the cellular biology results are in concordance with the previously obtained biochemical data, further supporting the notion that both wild type TFII-I as well as N90 TFII-I are capable of interacting with Btk in the cytoplasm.

**The First 90 Amino Acids of the N-terminal Region of TFII-I Are Sufficient in Mediating Dimeric Interactions with the Δ and with the β-Isomers of TFII-I**

Given that the first 90 amino acids in TFII-I were also indirectly implicated in its dimerization (14), we tested whether N90 would directly mediate dimerization of TFII-I isoforms. Thus, a GST pull-down assay was performed in order to test the ability of TFII-I-β and TFII-I-Δ isoforms to dimerize with the N90 mutant (Fig. 7). TFII-I-β, TFII-I-Δ, as well as ΔN90 (used as a negative control) were expressed as GFP fusion proteins in the presence of GST-tagged N90 or GST-tagged TFII-I-Δ (used as a positive control). N90 interactions with TFII-I isoforms were detected via anti-GFP immunoblotting. Input levels of total protein from whole cell lysates used in the GST pull-down were also included. The blot was stripped and re-probed with anti-GST to control for N90 or TFII-I-Δ GST-tagged protein levels. Results indicated that, as previously demonstrated, TFII-I-Δ was able to dimerize with itself (positive control). Moreover, both TFII-I-β and TFII-I-Δ were shown to interact with N90. As expected, our negative control showed that N90 failed to interact with the ΔN90 mutant. These findings suggest that N90 represents a dimerization domain that is sufficient in mediating interactions with different TFII-I isoforms as well as with Btk.

**Phosphorylation of Src-dependent Tyrosine Residues within TFII-I Is Not Required for TFII-I-Btk Complex Formation**

Recent work from our laboratory has established that phosphorylation at tyrosine residues Tyr248/249 and Tyr611 is required for Src-dependent transcriptional activation of TFII-I (13). Thus, we questioned whether these same Src-specific tyrosine phosphorylation sites within TFII-I were necessary for TFII-I-
**Phosphorylation of Src-dependent Tyrosine Residues within TFII-I**

Although Src-dependent phosphorylation-deficient mutants of TFII-I were shown to interact with Btk and to be phosphorylated by the latter, their transcriptional effects on the c-fos promoter upon co-expression with Btk needed to be tested in functional assays. Whole cell lysates of samples used in the luciferase reporter were analyzed via Western blotting; TFII-I protein levels were detected using anti-GST antibody (Fig. 9B). The immunoblot was stripped and re-probed with anti-HA to control for Btk expression levels (Fig. 9B). Our data indicate that the phosphorylation-deficient mutants of TFII-I exhibited similar effects on c-fos transcription relative to wild type TFII-I whether in the absence or in the presence of HA-tagged Btk. Subsequently, we assessed in reporter assays the effect of Btk on wild type or mutant TFII-I-mediated transcription of the c-fos promoter (Fig. 9A). Whole cell lysates of samples used in the luciferase reporter were analyzed via Western blotting; TFII-I protein levels were detected using anti-GST antibody (Fig. 9B). The immunoblot was stripped and re-probed with anti-HA to control for Btk expression levels (Fig. 9B). Our data indicate that the phosphorylation-deficient mutants of TFII-I exhibited similar effects on c-fos transcription relative to wild type TFII-I whether in the absence or in the presence of Btk (Fig. 9A). Indeed, as observed previously, overexpression of Btk induced an increase in transcription of the c-fos promoter in the presence of wild type TFII-I, and expression of Btk alone had no appreciable effects on the c-fos promoter (data not shown and Ref. 32). It might be argued that a slight decrease is observed in the Btk-dependent transcriptional activity of the Y611F+YY248/249FF mutant (M4) on the c-fos promoter (Fig. 9A). However, upon careful examination of the data, one can observe that this apparent “drop” in transcriptional activity is also accompanied by slightly lower levels in ectopic TFII-I-protein expression in this sample (Fig. 9B). Moreover, our data have been consistently reproducible and have been confirmed by other experiments of a similar kind. The data presented here demonstrate that the Src-dependent phosphorylation mutants of TFII-I did not abrogate Btk-mediated basal transcription of the c-fos promoter, suggesting that Btk does not target these sites under the conditions tested. The transcriptional effects are modest, but they represent basal levels of transcriptional activity given that the assay was conducted in the absence of any growth factor stimulation.

**DISCUSSION**

Extracellular signals are ultimately transduced to the nucleus to bring forth changes in cellular responses. In B cells, antigenic signals are transduced via surface immunoglobulins to activate initially a set of non-receptor tyrosine kinases. These events set the cellular machinery in motion such that signals finally reach the nucleus. Btk is one such kinase, and its activation is necessary for immune responses (6, 21, 22). Nevertheless, the exact pathways connecting Btk-mediated signaling to immune-specific gene expression are not fully understood. For instance, signal transducer and activator of transcription 5A directly interacts with and is a substrate of Btk (23). Moreover, it has been demonstrated that B cell signaling via Btk activates NF-κB by inducing phosphorylation of IκB (Btk does not directly act on NF-κB) (24–26). Although it appears that the Btk-NF-κB pathway controls B cell survival upon BCR signaling, proliferative responses could be controlled by an NF-κB-independent pathway. In addi-
to this important pathway, we have shown that Btk also activates the transcription factor TFII-I. In an uninduced (resting) state, TFII-I is retained in the B cell cytoplasm by direct interactions with Btk. Upon signaling through the BCR, TFII-I is tyrosine-phosphorylated in a Btk-dependent fashion and subsequently released from protein complex binding with Btk. Tyrosine-phosphorylated TFII-I translocates to the nucleus for gene activation. Given that TFII-I is a nuclear transcription factor and

![Fig. 8](image-url) The phosphorylation-deficient mutants and the ΔPPII mutant of TFII-I can interact with Btk and be tyrosine-phosphorylated upon Btk co-expression. A, GST-tagged wild type (WT) TFII-I and phosphorylation-deficient mutants of TFII-I as well as the ΔPPII mutant were co-expressed with HA-tagged Btk in COS-7 cells and subjected to whole cell extract immunoprecipitation (IP) with anti-HA antibody. M1, YY248/249FF; M2, Y277F; M3, YY248/249FF+Y277F; M4, YY248/249FF+Y611F; TFII-I interactions with Btk were detected by Western blotting (WB) using anti-GST antibody. WCE, whole cell extract input/WB lanes, 30 μg; IP lanes, 1000 μg. B, GST-tagged wild type TFII-I and phosphorylation-deficient mutants of TFII-I were co-expressed with HA-tagged Btk in COS-7 cells and subjected to a GST pull-down assay (PD). M1, YY248/249FF; M2, Y277F; M3, YY248/249FF+Y277F; M4, YY248/249FF+Y611F; M5, S631A (control). 1000 μg of whole cell lysates were used for the assay. Phosphotyrosine levels were assessed via Western blotting by using anti-phosphotyrosine (Anti-P-Tyr) antibody. The blot was stripped and re-probed with anti-GST antibody to monitor TFII-I levels. C, binding of c-Src to ΔPPII mutant of TFII-I is impaired. GST-tagged wild type TFII-I and the ΔPPII mutant of TFII-I were co-expressed with c-Src in COS-7 cells and subjected to a GST pull-down assay. 500 μg of whole cell lysates were used for the pull-down (PD) and 10 μg of whole cell extracts were used as input controls. TFII-I-c-Src interactions as well as c-Src expression levels (endogenous and ectopic) were visualized via Western blotting by using anti-c-Src antibody. The blot was stripped and re-probed with anti-GST antibody to monitor TFII-I levels.

![Fig. 9](image-url) Src-dependent phosphorylation-deficient mutants of TFII-I do not down-modulate c-fos promoter transcription in the absence or presence of Btk. A, wild type (wt) TFII-I and phosphorylation-deficient mutants of TFII-I were expressed with or without HA-Btk in COS-7 cells and subjected to a luciferase reporter assay using the c-fos promoter. Vector, pEBG; M1, YY248/249FF; M2, Y277F; M3, YY248/249FF+Y277F; M4, YY248/249FF+Y611F. B, Western blot analysis of equivalent volumes (50 μl) of whole cell lysates used in the luciferase reporter assay. Top panel, TFII-I expression levels were visualized via immunoblotting using anti-GST antibody. Bottom panel, the blot was stripped and re-probed using anti-HA to monitor Btk expression levels.
Btk is a cytoplasmic tyrosine kinase, it was necessary to mechanistically determine how these proteins interact with each other. We believe that a fuller understanding of this mechanism will provide a better appreciation of the Btk-TFII-I pathway and its importance for B cell signaling and gene regulation.

Btk is a non-receptor tyrosine kinase of the hematopoietic lineage, which belongs to the Tec family of protein-tyrosine kinases (Bmx, Itk, Tec, and Rlk/Txk) (17). In order to avoid endogenous effects of Btk, we chose to utilize COS-7 fibroblasts, which are devoid of Btk expression. Moreover, COS cells are also ideally suited for ectopic co-expression studies because recombinant proteins are expressed at high levels in these cells. Employing this expression system, we have identified key domains within TFII-I allowing it to interact with Btk. Furthermore, we have established that splice variants of TFII-I (α-, β-, and Δ-isofoms) are all capable of interacting with and being phosphorylated by Btk. The primary domain of interaction of TFII-I with Btk resides within the N-terminal region of TFII-I, containing residues 1–90. But surprisingly, we find that the putative leucine zipper motif comprised within these 90 amino acids is not required to mediate TFII-I-Btk complex formation because point mutations within the leucine zipper region do not abolish binding of TFII-I with Btk. The exact contact points between TFII-I and Btk within these 90 amino acids remain to be identified and are the subject for further study. We present evidence to indicate that the N90 region of TFII-I is required for its dimerization. Because dimerization appears to be a prerequisite for nuclear translocation of TFII-I (14), our data suggest that in a resting state, Btk retains TFII-I in the cytoplasm by preventing its dimerization (Fig. 10). We propose that upon antigenic signaling, TFII-I is phosphorylated in a Btk-dependent fashion, leading to its release from Btk. Activated (tyrosine-phosphorylated) Btk subsequently docks to the cytoplasmic membrane in a lipid-dependent process, where it plays an important role in calcium mobilization (27–30). Subsequently, tyrosine-phosphorylated TFII-I dimerizes and translocates to the nucleus where it effects activation of target genes (Fig. 10). Although unlikely, we cannot completely rule out the possibility that Btk interacts with the dimeric form of TFII-I.

The notion that the leucine zipper is important for dimerization of TFII-I is also consistent with the fact that the leucine zipper mutants appear to dimerize 2-fold less with wild type TFII-I than the wild type protein does with itself (data not shown). Noteworthy is the fact that although the N-terminal 90 amino acids of TFII-I contribute predominantly for its dimerization, TFII-I also possesses six I-repeats, which contain helix-loop-helix motifs that might serve to mediate protein-protein interactions. Indeed, these repeats also appear to provide secondary contact points for the dimerization of TFII-I (14). Nevertheless, our results indicate that isolated helix-loop-helix-containing repeats of TFII-I are incapable of interacting with Btk by themselves, and thus, the major contact points for Btk within TFII-I lie within the first 90 N-terminal amino acids.

One unexpected result arose from the mutant interaction studies with Btk, whereas the p70 mutant exhibited a comparable degree of interaction with Btk relative to wild type TFII-I, the p46 mutant, lacking an even greater portion of TFII-I at the C-terminal end, appeared to show an enhanced interaction with Btk relative to wild type TFII-I. Previous results from our laboratory (20) have shown that p70 naturally folds into a closed conformation, because it is inaccessible to limited protease digestion. Given the compactness of the p70 domain of TFII-I, we predict that p46 is forced to assume an artificially open conformation, possibly leading to an aberrant interaction of p46 with Btk. Current experiments are being conducted in our laboratory to determine if the C-terminal end of TFII-I putatively represents a weaker, secondary interaction site with Btk.

We and others (11, 12) have shown previously that TFII-I undergoes induced tyrosine phosphorylation upon growth factor signaling. We have mapped the major growth factor-dependent tyrosine phosphorylation sites contained within TFII-I (13). Nevertheless, it is not fully clear which is/are the kinase(s) that phosphorylate TFII-I at these specific sites. Although it has been shown that JAK2 can phosphorylate TFII-I at Tyr248 (31), we have shown by using site-specific phospho-antibodies that both the nuclear translocation and the tyrosine phosphorylation of TFII-I at Tyr248 remain intact in fibroblasts derived from Jak2−/− mice (data not shown). Moreover, in our hands, we observe a clear requirement for Src family kinases in mediating the phosphorylation of TFII-I at Tyr248 (13). By using transient overexpression studies in 293T cells, it has been shown that Tyr248 is also a Btk-dependent phosphorylation site (32). Whereas it is possible that under different conditions and in different cell lines, Tyr248 is phosphorylated by distinct tyrosine kinases, it is also likely that overexpression of Btk in 293T cells might aberrantly activate an Src family kinase. The fact that the YY248/249FF mutant still exhibits significant tyrosine phosphorylation as well as Btk-dependent transcriptional activity suggests that Btk does not phosphorylate this site under our conditions. However, it is possible that other tyrosine residues remaining intact in our mutant constructs (such as Tyr357 and Tyr462 (32)) might be phosphorylated by Btk under our conditions.

A related question thus emerges: can TFII-I be targeted by different kinases at distinct sites? This raises the possibility that different signaling pathways can simultaneously target TFII-I. Consistent with this notion, we show that c-Src and Btk can affect TFII-I through distinct and independent pathways given
that their interaction and phosphorylation sites differ. Whereas Btk interacts with TFII-I mostly through the first 90 N-terminal amino acids of TFII-I, c-Src interaction requires an intact PPII helix motif. Hence, we postulate that the transcription factor TFII-I possesses a unique capability of being simultaneously regulated by several independent signaling pathways.

Acknowledgments—We thank Dr. Venugopalan Cheriyath for the construction of the pEBG-PPII plasmid and for the initial data revealing the biochemical interactions between wild type TFII-I, ΔPPII, and c-Src. We are especially grateful to Dr. Andrew Chan and Dr. Chris Chiu for the pcDNA3.1/Zeo/FLAG-Btk plasmid and to Dr. David Rawlings for the anti-Btk antibody. We also thank Dr. Peter Brodeur for critical reading of the manuscript.

REFERENCES

1. Saouaf, S. J. S., Mahajan, R. B., Rowley, S. A., Kut, J., Fargnoli, A. L., Burkhart, S., Tsukada, O., Witte, O., and Bolen, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9524–9528
2. Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Kissak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J., Cooper, M., Conley, M., and Witte, O. (1993) Cell 72, 279–290
3. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, E., and Bentley, D. R. (1993) Nature 361, 226–233
4. Rawlings, D. J., Saffran, D., Tsukada, S., Largaespada, D., Grimaldi, J., Cohen, L., Mohr, R., Bazan, J., Howard, M., Copeland, N., Jenkins, N., and Witte, O. (1993) Science 261, 358–361
5. Thomas, J. D., Siders, P., Smith, C. I. E., Vorechovsky, I., Chapman, V., and Paul, W. E. (1993) Science 261, 355–358
6. Khan, W. N., Ali, F. W., Gerstein, R., Malynn, B., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kang, K., Rosen, F., and Sideras, P. (1995) Immunity 3, 263–269
7. Khan, W. N., Nilsen, A., Mizuguchi, E., Castigl, E., Forsell, J., Bhan, A., Geha, R., Siders, P., and Ali, F. (1997) Int. Immunol. 9, 395–405
8. Novina, C. D., Kumar, S., Bajpai, U., Cheriyath, V., Zhang, K., Pillai, S., Worts, H. H., and Roy, A. L. (1999) Mol. Cell. Biol. 19, 5014–5024
9. Yang, W., and Desiderio, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 604–609
10. Roy, A. L. (2001) Gene (Amst.) 274, 1–13
11. Novina, C. D., Cheriyath, V., and Roy, A. L. (1998) J. Biol. Chem. 273, 33443–33448
12. Kim, D.-W., Cheriyath, V., Roy, A. L., and Cochran, B. H. (1998) Mol. Cell. Biol. 18, 3310–3320
13. Cheriyath, V., Desgranges, Z. P., and Roy, A. L. (2002) J. Biol. Chem. 277, 22798–22805
14. Cheriyath, V., and Roy, A. L. (2001) J. Biol. Chem. 276, 8377–8383
15. Cheriyath, V., and Roy, A. L. (2000) J. Biol. Chem. 275, 26300–26308
16. Gao, J., Zeller, K. E., Ginsberg, M. H., Brugge, J. S., and Shattil, S. J. (1997) EMBO J. 16, 6414–6425
17. Smith, C., Islam, T., Mattson, P., Mohamed, A., Nore, B., and Vihinen, M. (2001) BioEssays 23, 436–446
18. Perez Jurado, L. A., Wang, Y. K., Peoples, R., Coloma, A., Cruces, J., and Franke, U. (1998) Hum. Mol. Genet. 7, 325–334
19. Wang, Y.-K., Perez Jurado, L., and Francke, U. (1998) Genomics 48, 163–170
20. Cheriyath, V., Novina, C., and Roy, A. (1998) Mol. Cell. Biol. 18, 4444–4454
21. Santos-Argumedo, L., Lund, F. E., Heath, A. W., Solvason, N., Wu, W., Grimaldi, J. C., Parkhouse, R. M., and Howard, M. (1995) Int. Immunol. 7, 163–170
22. Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kate, R. M., Fluckiger, A. C., Witte, O. N., and Kinnet, J. P. (1996) Science 271, 822–825
23. Mahajan, R. B., Vassilev, A., Sun, N., Ozer, Z., Mao, C., and Uckun, F. (2001) J. Biol. Chem. 276, 31216–31228
24. Bajpai, U., Zhang, K., Teutsch, M., Sen, R., and Wortis, H. (2000) J. Exp. Med. 191, 1735–1744
25. Petro, J., Rahman, S., Ballard, D., and Khan, W. (2000) J. Exp. Med. 191, 1745–1754
26. Sajio, K., Mecklenbrauker, I., Santana, A., Leitger, M., Schmedt, C., and Tarakhovsky, A. (2002) J. Exp. Med. 191, 1647–1652
27. Gou, B., Kato, R., Garcia-Lloret, M., Wahl, M., and Rawlings, D. (2000) Immunity 13, 243–253
28. Kang, S., Wahl, M., Chu, J., Kita, J., Kawakami, Y., Kato, R., Tabuchi, R., Tarakhovsky, A., Kawakami, T., Turck, C., Witte, O., and Rawlings, D. (2001) EMBO J. 15, 3562–3572
29. Chiu, C., Dallen, M., Ishii, M., Kurosaki, T., and Chan, A. (2002) EMBO J. 21, 6461–6467
30. Takata, M., and Kurosaki, T. (1996) J. Exp. Med. 184, 31–40
31. Kim, D.-W., and Cochran, B. H. (2001) Mol. Cell. Biol. 21, 3387–3397
32. Egloff, A., and Desiderio, S. (2001) J. Biol. Chem. 276, 27806–27815
