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Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement

(Lyn/Blk/Btk/Syk/Jak)

SANDRA J. SAOUAF*, SANDEEP MAHAJAN*, R. BRUCE ROWLEY*, STEPHANIE A. KUT*, JOSEPH FARGNOLI*, ANNE L. BURKHARDT*, SATOSHI TSUKADA†, OWEN N. WITTE†, AND JOSEPH B. BOLEN*‡

*Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543; and †Howard Hughes Medical Institute, University of California, Los Angeles, CA 90024

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ABSTRACT **We evaluated in WEHI 231 B cells the time-dependent responses of Lyn, Blk, Btk, Syk, and three members of the Jak family of protein tyrosine kinases following antibody-mediated surface engagement of the B-cell antigen receptor. Our results show that the enzyme activities of Lyn and Blk were stimulated within seconds of antigen receptor engagement and correlated with the initial tyrosine phosphorylation of the Igα and Igβ subunits of the B-cell antigen receptor. Btk enzyme activity was also transiently stimulated and was maximal at ≈5 min after B-cell receptor surface binding. Syk activity gradually increased to a maximum at 10–30 min following receptor ligation and was found to parallel the association of Syk with the tyrosine phosphorylated Igα and Igβ subunits of the receptor. While the specific activities of the Jak1, Jak2, and Tyk2 protein tyrosine kinases were unaltered following B-cell receptor ligation, the abundance of Jak1 and Jak2 were increased 3- to 4-fold within 10 min of receptor engagement. These results demonstrate that multiple families of non-transmembrane protein tyrosine kinases are temporally regulated during the process of B-cell antigen receptor-initiated intracellular signal transduction.**

The B-cell antigen receptor (BcR) is composed minimally of surface immunoglobulin (sIg) noncovalently associated with a pair of Igα/Igβ disulfide-linked heterodimers (1, 2). The sIg acts as the antigen binding ectodomain of the receptor while Igα/Igβ functions to couple sIg with intracellular signal transducing enzymes (3–6). Among the enzymes functionally coupled to sIg through Igα/Igβ are several non-transmembrane protein tyrosine kinases (PTKs) including members of the Src family as well as Syk (7–12). Appropriate surface ligation of the BcR results in the activation of B-cell PTKs and the phosphorylation of lymphocyte proteins on tyrosine residues including Igα and Igβ (13–16). Phosphorylation of a pair of conserved tyrosine residues in the cytoplasmic domain of Igα/Igβ is required for successful propagation of BcR signal transduction (4, 17). This conserved sequence motif YXXLXXXXXXXXYXXI/L (Y = tyrosine, I = isoleucine, L = leucine, X = any amino acid) or tyrosine activation motif (TAM) (18) is present in the cytoplasmic signal transducing sequences of multichain immune recognition receptors (19, 20).

While several members of the membrane-associated Src family have been shown to be enzymatically activated following BcR engagement, current evidence suggests that $p53/p56^{lyn}$ (Lyn) and $p56^{blk}$ (Blk) may have pivotal roles in this process. Blk is a B-lymphocyte-specific enzyme (21) whose expression is developmentally correlated with the expression of Igα and Igβ in immature B lymphocytes (22). BcR signal transduction has been shown to be compromised when Blk expression was suppressed using anti-sense blk-specific oligonucleotides (23). While Lyn is expressed in a variety of cells, it is activated following BcR crosslinking (7–9) and has been shown in B cells to associate with the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI-3 kinase) (24) leading to elevation in PI-3 kinase activity (25). Importantly, recent studies using targeted disruption of the *lyn* locus to generate *lyn* null avian B cells demonstrated that Lyn is needed for optimal BcR signaling (26).

The 72-kDa Syk and the 70-kDa ZAP-70 (Zap) represent a family of cytoplasmic PTKs that are structurally novel due to the presence of two tandem src homology 2 (SH2) domains amino terminal to the enzymes' catalytic domain (27, 28). Zap association with tyrosine phosphorylated TAMs present in various T-cell receptor subunits has been shown to be important for T-cell receptor-mediated signaling events (29). B cells deficient in expression of Syk have been shown to be severely compromised in BcR-initiated tyrosine phosphorylation of phospholipase C-γ2 as well as in inositol 1,4,5-trisphosphate generation and calcium mobilization (26).

Btk, Itk, and Tec comprise a family of cytoplasmic PTKs that possess amino-terminal pleckstrin homology domains followed by single SH3, SH2, and catalytic domains (30). Among this group of PTKs, the 77-kDa Btk has been shown to be important for B-cell development and function. The gene encoding the Btk enzyme represents the defective gene in human X chromosome-linked agammaglobulinemia as well as in murine X chromosome-linked immunodeficiency (31–34). However, a role for Btk in BcR signaling has not been identified.

The Jak family of PTKs represents a group of cytoplasmic enzymes that have been shown to be critical for signal transduction initiated through ligand binding to a number of multisubunit cytokine receptors (35). Whether Jak-related kinases also participate in multichain immune recognition receptor signaling is not known.

MATERIALS AND METHODS

Cell Growth and Activation. The murine B-cell line WEHI 231 was used for all experiments. For BcR activation the cells were adjusted to 1×10^7 per ml and stimulated with 20 μg of goat anti-mouse IgM (anti-μ) per ml (Sigma) (7). The cells

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Abbreviations: anti-μ, goat antisera to murine IgM; APT, anti-phosphotyrosine; BcR, B-cell antigen receptor; GST, glutathione-*S*-transferase; PTK, protein tyrosine kinase; sIg, surface immunoglobulin; SH, src homology; TAM, tyrosine activation motif.

‡To whom reprint requests should be addressed at: Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543-4000.

were lysed in a Nonidet P-40 lysis buffer (7) as a function of time from 5 sec to 60 min following addition of the stimulating antibody.

Antibodies. Rabbit antisera directed against sequences unique to Lyn, Blk, Btk, and Syk have been described (7, 31, 36, 37). Rabbit antisera to Jak1 and Jak2 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit antisera to Tyk2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Biochemical Analyses. Immunoprecipitation, immune-complex protein kinases assays, and immunoblotting have all been described (38, 39). For some immune-complex protein kinase assays, a glutathione-*S*-transferase (GST) fusion protein containing the cytoplasmic domain of the BcR Igα subunit was used as an exogenous substrate (5 μg per reaction mixture). Quantitation of phosphorylated reaction products was performed by analysis with a Molecular Dynamics PhosphorImager using ImageQuant software.

RESULTS

Protein Tyrosine Phosphorylation Following BcR Ligation. WEHI 231 cells were stimulated and lysed as a function of time from 5 sec to 60 min following addition of anti-μ serum and the lysates were examined by anti-phosphotyrosine (APT) immunoblotting (Fig. 1*A*). By using this method of BcR activation and analysis, an overview of the timing and pattern of responding protein phosphorylation was established.

The TAMs of the Igα and Igβ subunits of the BcR have been reported to be tyrosine phosphorylated in response to BcR ligation (15, 16). The results shown in Fig. 1*B* demonstrate that in WEHI 231 cells tyrosine phosphorylation of Igα could be detected as soon as 5 sec following BcR ligation and continued to increase over the course of the experiment. As the abundance of Igα was not altered during this experiment (Fig. 1*C*), the results indicate that the changes in Igα APT reactivity reflect increased Igα tyrosine phosphorylation.

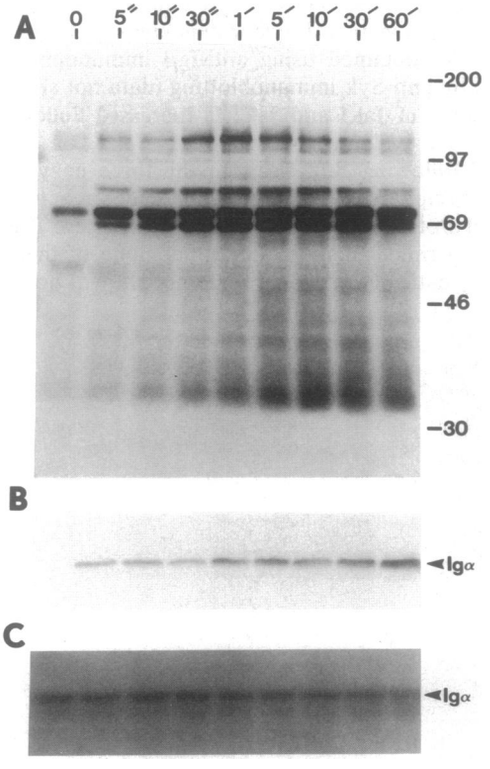

FIG. 1. Time course of WEHI 231 cell protein tyrosine phosphorylation and Igα tyrosine phosphorylation. WEHI 231 cells were stimulated with anti-μ antibody and lysed as a function of the indicated times. Total cell protein tyrosine phosphorylation was detected by APT immunoblotting (*A*) or cell lysates were immunoprecipitated with anti-Igα and immunoblotted with either APT (*B*) or anti-Igα (*C*). Positions of prestained molecular mass markers (in kDa) (GIBCO/BRL) and Igα are indicated.

Activation of Blk, Lyn, Btk, and Syk. WEHI 231 cells were stimulated by addition of anti-μ and lysates were prepared over the time course established above. As shown in Fig. 2, the protein kinase activities of both Blk (Fig. 2*A*) and Lyn (Fig. 2*B*) were elevated within 5 sec of BcR ligation. In these autophosphorylation assays, Blk activity peaked at ≈10 sec and was maintained at an elevated level for about 30 min. The abundance of Blk was not altered over the course of these experiments (Fig. 2*E*), indicating that the changes in enzyme activity likely reflected altered specific activity. Blk activity was found to roughly parallel the reactivity of Blk with APT antibodies (Fig. 2*I*). Lyn activity was found to peak at ≈5–10 sec following addition of anti-μ and was maintained at elevated levels for about 10 min. Lyn activity was found to decrease dramatically at 30–60 min to levels below that observed prior to anti-μ addition. As shown in Fig. 2*F*, the abundance of Lyn was constant over the first 10 min following BcR ligation and then decreased ≈3- to 4-fold by 60 min. Thus, the initial increases in Lyn protein kinase activity appear to represent changes in enzyme specific activity, while the diminished Lyn protein kinase activity detected at the later times in these experiments likely reflects the decreased abundance of the enzyme. Lyn protein kinase activity was found to parallel the reactivity of the enzyme with APT antibodies (Fig. 2*J*).

The protein kinase activity of Btk was found to increase following addition of anti-μ (Fig. 2*C*). Elevated Btk activity could be detected as early as 10–30 sec after anti-μ addition with the peak of Btk autophosphorylating activity (4-fold over time 0) found at ≈5 min. The low level of Btk activity observed at 60 min in this experiment was not reproducible. Generally, Btk protein kinase activity returned by 60 min to approximately the same level of activity as that observed at time 0 (see Fig. 3). In these experiments, the level of Btk protein was not detectably altered (Fig. 2*G*), thereby suggesting that the changes in protein kinase activity were the result of altered specific activity. We also found that the enzyme activity of Btk clearly paralleled the reactivity of Btk with APT antibodies (Fig. 2*K*), suggesting that Btk protein kinase activity is modulated by tyrosine phosphorylation.

The protein kinase activity of Syk was also elevated following BcR engagement (Fig. 2*D*). Syk kinase activity was increased at about 30 sec after addition of anti-μ and increased to a maximum (3-fold) at ≈10–60 min. The abundance of Syk was not altered over the course of these experiments (Fig. 2*H*). Syk was also found to increase significantly in reactivity with APT antibodies following BcR ligation (Fig. 2*L*). In this case, changes in Syk APT reactivity were observed as early as 5 sec following addition of anti-μ and peaked between 5 and 60 min.

As shown in Fig. 3, the suggested order of PTK activation indicated by the measurement of enzyme autophosphorylation was clearly confirmed by assessing the capacity of Blk, Btk, and Syk to phosphorylate an exogenous substrate. In this case, the common exogenous substrate used was a chimeric protein consisting of GST coupled to the cytoplasmic domain of Igα. The results of this experiment (Fig. 3*B*) demonstrate that Blk was activated within 5 sec following addition of anti-μ. This activation was transient with the peak of activity (5-fold) occurring between 5 and 10 sec following receptor engagement. In contrast to the immediate activation of Blk, the transient activation of Btk (3-fold) in this assay was found to peak 5–10 min following BcR ligation (Fig. 3*D*). As found in the autophosphorylation analyses, Syk protein

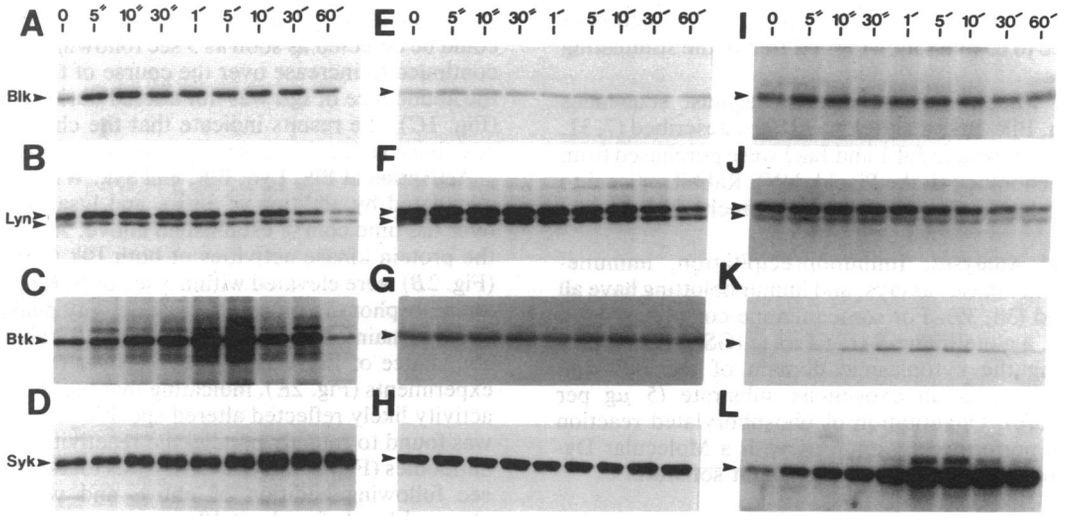

FIG. 2. Time course of Blk, Lyn, Btk, and Syk enzyme activation and tyrosine phosphorylation. WEHI 231 cells were stimulated with anti-μ, lysed at the indicated times, and the cell lysates were immunoprecipitated with antibodies specific for the indicated PTKs. The protein kinase activities of the immunoprecipitated PTKs were evaluated in immune-complex autophosphorylation assays (*A–D*). The relative abundance of the PTKs was estimated by anti-PTK immunoblotting (*E–H*) and the tyrosine phosphorylation state of the enzymes was estimated by APT immunoblotting (*I–L*). Positions of $p56^{blk}$ (Blk) (*A*, *E*,, and *I*), $p53/p56^{lyn}$ (*B*, *F*, and *J*), $p77^{btk}$ (*C*, *G*, and *K*), and $p72^{syk}$ (*D*, *H*, and *L*) are indicated.

kinase activity was observed to increase gradually following BcR ligation and peak at ≈10–60 min (Fig. 3*F*). The peak change in Syk protein kinase activity was found to be 3- to 4-fold.

Association of Syk with Igα/Igβ. We tested whether these enzymes would associate with the BcR signaling components under the conditions used to lyse the cells for analysis of PTK enzyme activity. Under these lysis conditions, we were unable to demonstrate stable association of Lyn, Blk, or Btk with Igα/Igβ. However, Syk was found to associate with Igα/Igβ following BcR engagement. As shown in Fig. 4*A*, several tyrosine phosphorylated proteins coimmunoprecipitating with Syk could be observed following anti-μ addition. Prominent among these was a protein that was the same size as Igα and that possessed a tyrosine phosphorylation profile similar to that established for Igα. To test for the association of these two proteins directly, Syk immunoprecipitates were subjected to immunoblotting with anti-Igα antibodies and, conversely, Igα immunoprecipitates were subjected to immunoblotting with anti-Syk antibodies. The results of this experiment (Fig. 4 *B–E*) demonstrate an increase in Syk:Igα complex formation as a function of time following BcR ligation. The kinetics of this association paralleled the tyrosine phosphorylation of both Igα and Syk as well as the changes in the kinetics of Syk protein kinase activity. Similar results were obtained using anti-Igβ immunoprecipitation followed by anti-Syk immunoblotting (data not shown).

Abundance of Jak1 and Jak2 Is Increased Following BcR Ligation. To evaluate whether Jak1, Jak2, or Tyk2 was activated following BcR ligation, the three enzymes were immunoprecipitated and their abundance, reactivity with APT, and protein kinase activities were assessed. The results of this experiment demonstrated that none of these PTKs were enzymatically activated (data not shown) nor tyrosine

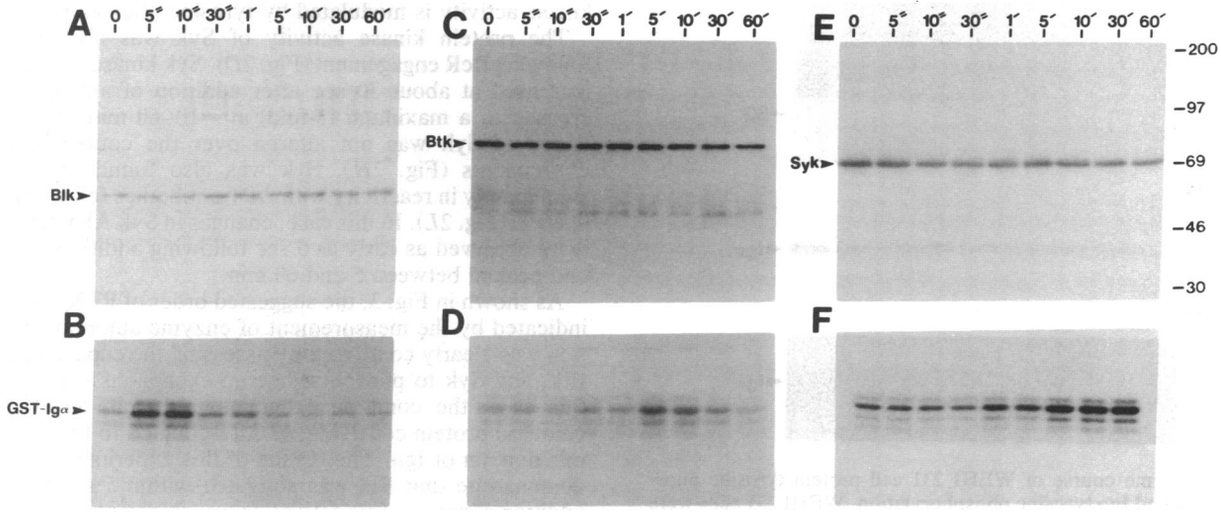

FIG. 3. Time course of Blk, Btk, and Syk enzyme activation for an exogenous substrate. WEHI 231 cells were stimulated with anti-μ, lysed at the indicated times, and the cell lysates were immunoprecipitated with antibodies specific for the indicated PTKs. The abundance of the Blk (*A*), Btk (*C*), and Syk (*E*) in the immune-complexes was assessed by PTK immunoblotting. The protein kinase activities of Blk (*B*), Btk (*D*), and Syk (*F*) in parallel immune-complexes were evaluated using GST-Igα as an exogenous substrate. Positions of Blk, Btk, Syk, GST-Igα, and prestained molecular mass markers (in kDa) are indicated.

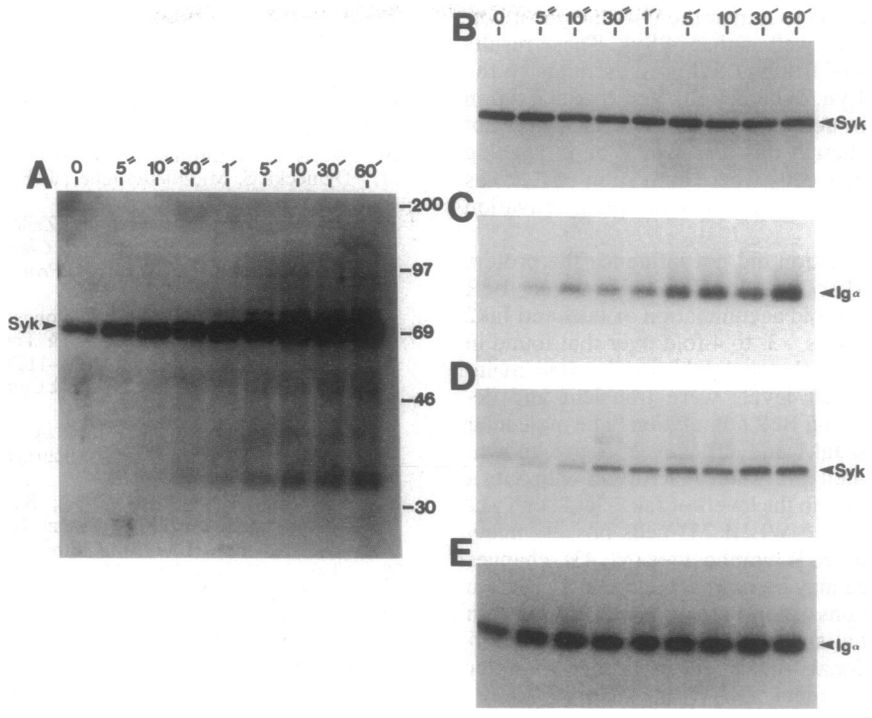

FIG. 4. Time course of Syk tyrosine phosphorylation and association with Igα. WEHI 231 cells were stimulated with anti-μ and the cells were lysed at the indicated times. The cell lysates were immunoprecipitated with antibodies to Syk (*A–C*) or antibodies to Igα (*D* and *E*). The immunoprecipitated proteins were fractionated on SDS/polyacrylamide gels and immunoblot analysis was conducted using APT (*A*), anti-Syk (*B* and *D*), or anti-Igα (*C* and *E*). Positions of Syk, Igα, and prestained molecular mass markers (in kDa) are indicated.

phosphorylated (Fig. 5 *B*, *D*, and *F*). However, the abundance of Jak2 (Fig. 5*A*) and Jak1 (Fig. 5*C*) was increased 3- to 4-fold within 10 min of BcR ligation. Whereas the elevated abundance of Jak2 was maintained through 60 min (Fig. 5*A*), the abundance of Jak1 decreased almost to control levels by this time (Fig. 5*C*). The abundance of Tyk2 was unchanged in these experiments (Fig. 5*E*).

DISCUSSION

The results of these assays indicate that different classes of B-cell PTKs are activated in a time-dependent manner following BcR surface ligation. Members of the Src family of PTKs such as Blk and Lyn appear to be activated very early, followed by activation of Btk and the later activation of Syk. These experiments suggest that Blk and/or Lyn may be the initiating PTKs in a cascade that contributes to B-cell activation.

The concept that Blk and Lyn participate in the initial signal generated from BcR ligation is consistent with previous studies in which Blk and Lyn activation has been examined (7–9) and in other experiments in which their expression has been compromised in B cells, leading to severely suppressed signaling initiated from the BcR (23, 26). This idea is also consistent with observations made in T cells in which the Src family member Lck has been shown to be an early response enzyme that is required for T-cell receptor signal transduction (29, 40). The localization of Blk and Lyn at the inner portion of the plasma membrane (41) places these enzymes in the appropriate subcellular location to potentially respond to surface ligation of the BcR. Previous observations that both Blk and Lyn can weakly associate with components of the BcR in nonactivated B cells (7) also support this possibility. The early activation of Blk and Lyn in B cells was found to coincide with the initial tyrosine phosphorylation of Igα. In other studies we have found that phosphorylation of Igβ demonstrates similar kinetics (data not shown).

Activation of Btk and Syk was found to peak following that of the Src enzymes. Btk displayed an activation maximum at ≈5 min following receptor ligation and Syk was most active at later times. Tyrosine phosphorylation of both enzymes was found to correlate roughly with their enzymatic activation. Syk, but not Btk, was found to associate with the Igα/Igβ BcR subunit following BcR ligation. The association between

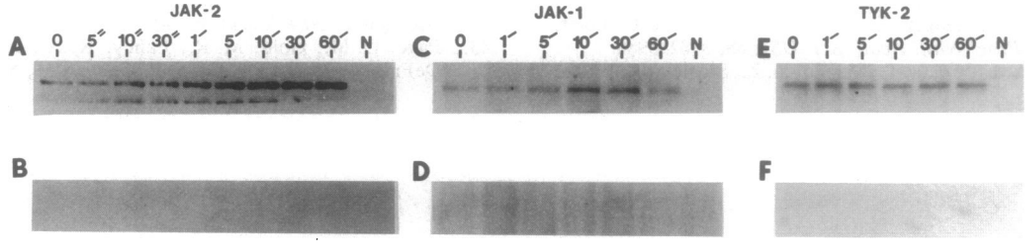

FIG. 5. Evaluation of Jak1, Jak2, and Tyk2 protein levels and tyrosine phosphorylation. WEHI 231 cells were stimulated with anti-μ, lysed at the indicated times, and the cell lysates were immunoprecipitated with antibodies specific for Jak2 (*A* and *B*), Jak1 (*C* and *D*), or Tyk2 (*E* and *F*). The lanes marked N are normal rabbit serum. The immunoprecipitated proteins were fractionated on SDS gels and the abundance of the enzymes was estimated by immunoblotting with anti-Jak2 (*A*), anti-Jak1 (*C*), or anti-Tyk2 (*E*). The tyrosine phosphorylation level of the enzymes was evaluated by APT immunoblotting (*B*, *D*, and *F*). Positions of Jak1, Jak2, and Tyk2 are indicated.

Igα/Igβ and Syk was found to mirror the tyrosine phosphorylation state of Igα/Igβ as well as that of Syk. These results suggest that activation of Btk and Syk is subsequent to the activation of Blk and Lyn. Whether Btk and Syk activation is dependent upon prior activation of Blk and/or Lyn is not clearly established, although this idea would be in keeping with previous results in T cells where activation of Lck has been demonstrated to be necessary for subsequent activation of Zap (29, 40).

We found that BcR ligation did not influence the protein kinase activities of Jak1, Jak2, or Tyk2. However, BcR ligation did result in the rapid accumulation of Jak1 and Jak2 in WEHI 231 cells to levels ≈3- to 4-fold over that found in nonactivated cells. While elevated Jak2 levels were maintained, the increased Jak1 levels were transient and decreased by 60 min following BcR stimulation. The molecular basis for the change in Jak1 and Jak2 levels is not known. Stimulation of other B-cell lines such as RAMOS with anti-μ did not result in alterations in the levels of Jak1, Jak2, or Tyk2 (unpublished results). Since WEHI 231 cells possess many characteristics of immature B lymphocytes (42, 43), changes in Jak family abundance may be representative of a program of BcR-induced alterations at this point of B-cell ontogeny in preparation for potential subsequent B-cell–cytokine interactions. Clearly, additional studies will be needed to better characterize this phenomenon.

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