Signal Transducer and Activator of Transcription-3 (STAT3) Is Constitutively Activated in Normal, Self-renewing B-1 Cells but Only Inducibly Expressed in Conventional B Lymphocytes

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Summary

Cytokine and growth factor receptor engagement leads to the rapid phosphorylation and activation of latent, cytosolic signal transducers and activators of transcription (STAT) proteins, which then translocate to the nucleus where they regulate transcriptional events from specific promoter sequences. STAT3 expression in particular has been associated with Abl, Src, and HTLV-1 transformation of normal cells. B-1 lymphocytes are self-renewing, $CD5^+$ B cells that display a propensity for malignant transformation and are the normal counterpart to human chronic lymphocytic leukemias. Further, B-1 cells are characterized by aberrant intracellular signaling, including hyperresponsiveness to phorbol ester PKC agonists. Here we demonstrate that B-1 lymphocytes constitutively express nuclear activated STAT3, which is not expressed by unmanipulated conventional (B-2) lymphocytes. In contrast, STAT3 activation is induced in B-2 cells after antigen receptor engagement in a delayed fashion (after 3 h). Induction of STAT3 is inhibited by both the serine/threonine protein kinase inhibitor H-7 and the immunosuppressive drug rapamycin and requires de novo protein synthesis, demonstrating novel coupling between sIg and STAT proteins that differs from the classical paradigm for STAT induction by cytokine receptors. The inability of prolonged stimulation of conventional B-2 cells with anti-Ig, a treatment sufficient to induce CD5 expression, to result in sustained STAT3 activation suggests that STAT3 is a specific nuclear marker for B-1 cells. Thus, STAT3 may play a role in B cell antigen-specific signaling responses, and its constitutive activation is associated with a normal cell population exhibiting intrinsic proliferative behavior.

The 67-kD pan–T cell surface glycoprotein, CD5, was first detected on the surface of human and murine B cell tumors and subsequently found to specify a subset of normal B lymphocytes in both species (1). $CD5^+$ (or B-1) B lymphocytes are mature B cells that predominate early in life, decline in relative number as the animal matures, and, in mice, become confined to the peritoneal cavity, with few, if any, present in the peripheral lymph nodes (2). Functionally, B-1 cells contribute a disproportionally large fraction of serum Ig, specifically of the μ, α, and γ classes. These Igs are noted to express germline encoded specificities, with little somatic mutation and N-insertion and may be involved in the regulation of idiotype expression (3, 4).

B-1 cells have been linked to both autoantibody production and the pathogenesis of autoimmune disease as well as malignancy (2, 5). $CD5^+$ B cells have been found to be enriched sources of autoantibody-producing cells specific for various self-antigens, and several mouse strains that develop autoimmune pathology have elevated numbers of splenic and peritoneal CD5 B cells (6, 7). Adoptive transfer experiments have demonstrated that B-1 cells have self-renewing capacity (8), and in vitro, these cells are readily immortalized in culture without the use of exogenously induced transformation (9). Coupled with their hyperresponsiveness to PMA stimulation and their inability to enter S phase after sIg cross-linking (10, 11), these observations suggest that B-1 cells differ from B-2 cells in their biochemical makeup in ways that may contribute to autoantibody secretion and unregulated growth.

Signal transducers and activators of transcription (STAT)[1] proteins were first characterized by studying signaling in re-

[1]*Abbreviations used in this paper:* CHX, cycloheximide; CNTF, ciliary neurotrophic factor; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C; SIE, *sis*-inducible element; SIF, *sis*-inducible factor; STAT, signal transducers and activators of transcription; TI-2, T cell–independent type II.

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sponse to interferon and have since been implicated in cellular responses to a plethora of cytokines and growth factors (12, 13). STAT signaling involves the activation of the JAK/tyk family of tyrosine kinases that are believed to be associated with unliganded cytokine receptors and to phosphorylate latent cytoplasmic STAT proteins upon ligand binding (14). Phosphorylated STATs dimerize via interactions between their SH2 domains (15), allowing nuclear translocation and DNA binding activity specific for distinct sequence elements in cytokine and growth factor–stimulated genes. We have previously shown that mitogenic stimulation through surface Ig in B-2 cells induces the activation of STAT proteins (16). This observation, coupled with the association of STAT3 with abnormal cell growth and transformation (17–20), led us to compare the status and activational responses of STAT3 proteins in B-1 and B-2 cells. Our results indicate that the nuclear expression of activated STAT proteins differs between B-1 and B-2 cells and that the STAT protein profile may be a distinguishing molecular feature of the B-1 cell phenotype.

Materials and Methods

Animals. Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed at least 1 wk before experimentation. Mice were cared for and handled at all times in accordance with National Institutes of Health and institutional guidelines.

B Cell Purification. B-1 lymphocytes were prepared by negative selection from peritoneal wash-out cells as previously described (21). B-2 cells were purified from spleen cells of 8–12-wk-old naive mice by depletion of T cells using treatment with anti–Thy 1.2 antibody plus rabbit complement and depletion of macrophages by overnight culture on plastic petri dishes, as previously described (22). RBC and nonviable cells were removed by sedimentation over Lympholyte M (Cedarlane, Ontario, Canada). The resulting B cells were cultured at 37°C with 5% CO_2 in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% heat inactivated fetal bovine serum (Sigma Chem. Co., St. Louis, MO), 10 mM Hepes (pH 7.2), 50 μM 2-ME, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. In some experiments, B-1 cells were prepared in the presence of serum-free RPMI 1640 medium containing 1% bovine serum albumin and cultured in serum-free AIM-V medium (GIBCO BRL, Gaithersburg, MD).

Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear extracts from untreated or stimulated primary B cells were prepared using 430 mM NaCl at pH 7.9 as previously described (22), except that 1 mM sodium orthovanadate was added to all extraction buffers. Protein concentrations were determined by the Bradford method (23) (Bio-Rad, Hercules, CA). Nuclear extracted protein was incubated with ^{32}P-labeled double-stranded oligonucleotide containing the high-affinity SIE (m67) derived from the *c-fos* gene (24) (5′GTGCAT**TTCCCGTAA**ATCTTGTCTACAATTC3′) for 20 min before electrophoresis on nondenaturing 5% polyacrylamide gels. Binding reactions contained 1 μg poly(dI-dC) and 2.0 μg salmon sperm DNA. For competition analysis, 20-fold excess unlabeled SIE or NF-AT oligonucleotide (25) was added to binding reactions before addition of nuclear protein and electrophoresis. Supershift/immunoinhibition analysis was performed by addition of 1 μl anti-p91N or antiphosphotyrosine^{701}STAT1 for an additional 30 min at 4°C after the 20-min electrophoretic mobility shift assay (EMSA) binding reaction.

Western Blotting. Nuclear extracted protein (5 μg) was resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and blocked with 5% nonfat powdered milk in wash buffer (20 mM Tris, pH 7.6, 0.14 M NaCl, and 0.1% Tween-20; TBS-T) overnight at 4°C. The nitrocellulose filters were then probed with antiphosphotyrosine^{701}STAT1 (26), or with antiphosphotyrosine^{705} STAT3 antiserum, generated by immunization of rabbits with a synthetic peptide containing amino acids 696-709 of human STAT3, with phosphotyrosine at position 705, which was conjugated to bovine serum albumin. Blots were incubated for 1 h at room temperature with antibody in 3% BSA–TBS-T. After washing, blots were developed by ECL.

Shift-Western. EMSA was performed as described above except 5 μg of nuclear extracts were added to 1.5× the normal amount of labeled oligonucleotide and electrophoresed on 5% polyacrylamide gels. SIE-binding proteins were separated from retarded labeled oligonucleotide by electrophoretic transfer from the native gel to nitrocellulose paper. Labeled oligonucleotide was detected bound to DE-81 paper (Whatman, Hillsboro, OR) placed under the nitrocellulose filter and on top of Whatman filter paper. After transfer, the nitrocellulose filters were blocked and Western blotted as above and the DE-81/Whatman filters were dried for 10 min before autoradiography.

Reagents. $F(ab')_2$ fragments of goat anti–mouse IgM (Jackson Immunoresearch, Inc., West Grove, PA) were used at a concentration of 15 μg/ml. PMA (Sigma) was used at 100 ng/ml. Cells were stimulated with murine recombinant IFN-γ (5 ng/ml) or IL-6 (1,000 U/ml), both from Genzyme (Cambridge, MA). Rabbit antiserum to the NH_2-terminal domain of p91 (anti-p91N) was the kind gift of Dr. C. Schindler (Columbia University, New York). Rabbit antiserum specific for only the tyrosine-phosphorylated form of STAT1 was generated by immunization of rabbits with a peptide designed from the STAT1α protein containing phosphorylated Tyr 701 (26). Rapamycin was used at 20 ng/ml, dissolved in ethanol and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) and N-(2′-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004) (LC Laboratories, Woburn, MA) were used at 25 μM.

Results and Discussion

To determine whether the unusual growth characteristics of B-1 cells are accompanied by differences in the regulation of STAT proteins, we compared STAT DNA-binding activities between resting B-1 and B-2 cells. Nuclear extracts from untreated B-1 cells formed protein–DNA complexes with the high-affinity *sis*-inducible element (SIE) of the *c-fos* gene (27), a recognized STAT-binding site (1), as detected by EMSA (Fig. 1 *A*). The major B-1 cell–specific SIE-binding activity was observed to co-migrate with the IL-6–stimulated *sis*-inducible factor (SIF) A binding complex, with a smaller amount co-migrating similarly to the IFN-γ–induced SIF C complex (24, 28–30). In contrast, no SIF A, and little SIF C activity was detected in nuclear extracts obtained from unmanipulated B-2 cells.

The B-1 cell complexes were competed by unlabeled SIE-containing oligonucleotide but not by the consensus binding site for the nuclear factor of activated T cells (NF-AT) (Fig. 1 *B*), indicating that these complexes are specific for the SIE. The constitutive expression of SIF complexes in

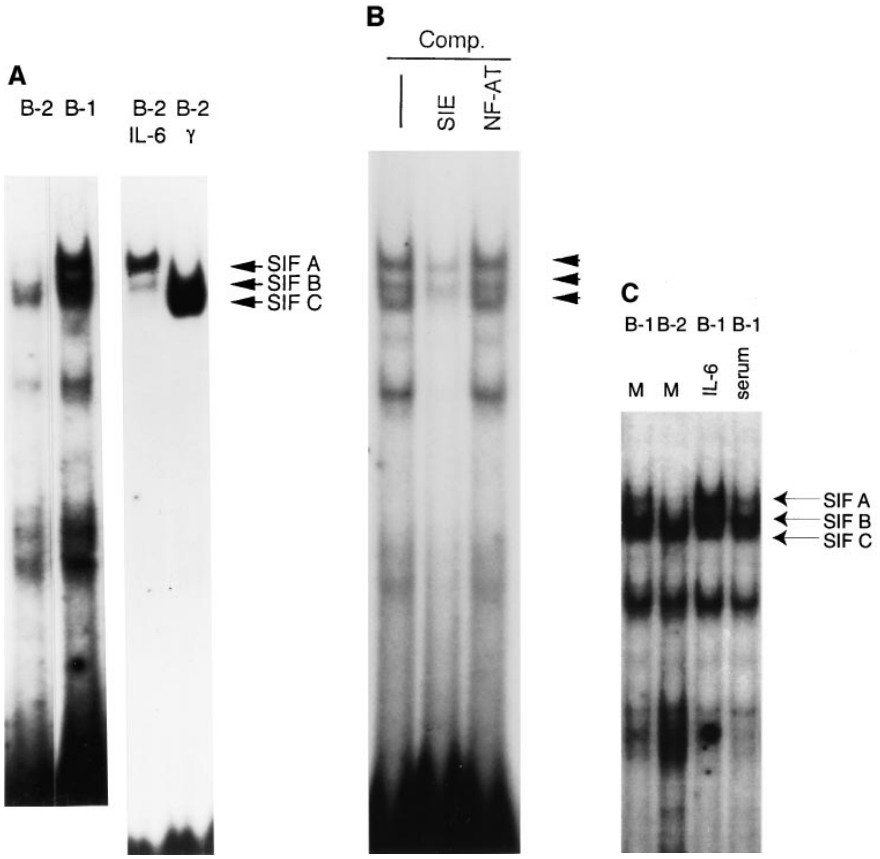

Figure 1. Constitutive expression of specific nuclear SIE-binding activity that comigrates with IL-6–induced SIF A in B-1 cells. (*A*) EMSA analysis of nuclear extracts prepared from B-2 cells (15×10^6) incubated in medium alone or treated with IL-6 (1,000 U/ml for 15 min; Genzyme, Cambridge, MA) or IFN-γ (γ; 5 ng/ml for 15 min; Genzyme), or B-1 cells (7.5×10^6) incubated in medium alone. Nuclear extracts were incubated with radiolabeled oligonucleotide containing the high-affinity *c-fos* SIE sequence (28). Arrows indicate the positions of STAT3 homodimers (*SIF A*), STAT3–STAT1 heterodimers (*SIF B*), and STAT1 homodimers (*SIF C*). (*B*) Specific binding activity of the constitutive B-1 SIE-binding complex. Competition analysis was performed on nuclear extracts from untreated B-1 cells in an EMSA using 20-fold excess unlabeled SIE or NF-AT (control) oligonucleotides. Arrows indicate the positions of SIE-binding complexes containing SIF A, B, and C. (*C*) Serum does not induce SIF A in B-1 cells. SIE–EMSA analysis of nuclear extracts prepared from B-1 cells purified and cultured in the presence of serum-free medium alone (lane *1*), or in serum-free medium plus IL-6 (1,000 U/ml for 15 min; lane *3*), or subsequent resuspension in serum-containing RPMI medium for 15 min (lane *4*). B-2 cells (lane *2*) were cultured in serum-containing RPMI medium alone.

B-1 cells was not the result of serum induction in vitro, as these complexes were still formed from nuclear extracts prepared from B-1 cells purified and cultured in serum-free medium and were not inducible upon subsequent serum treatment (Fig. 1 *C*). Antibody to the NH_2-terminal region of STAT1α (anti-p91N), which has been shown to recognize STAT3 induced by ciliary neurotrophic factor (CNTF) (31), disrupted SIF A induced by IL-6 in B cells (Fig. 2 *A*). The constitutively expressed B-1 SIF A–like complex was also disrupted by anti-p91N but not by an antibody to phosphotyrosine^{701}STAT1 (Fig. 2 *A*). Anti-p91N formed supershifted complexes with SIF A in both IL-6–stimulated B cells and untreated B-1 cells which were apparent in longer exposures and showed identical electrophoretic mobilities; antisera to STAT 4, 5, or 6 failed to react with the B-1 cell SIF A complex in EMSA supershift assays or with B-1 cell nuclear extracts in Western blotting experiments, and antibody that supershifted STAT1 activated by IFN-γ immunoinhibited/supershifted only a small amount of the SIF C complex of B-1 cells (data not shown). Because STAT3 is only known to form homodimers or to heterodimerize with STAT1, these results strongly suggest that B-1 cells differ from B-2 cells in the basal nuclear expression of STAT3 homodimers that comprise the SIF A nucleoprotein complex.

To assess the contribution of phosphorylated STAT3 to the SIF A complex constitutively present in B-1 cells, we performed Shift–Western experiments, using nuclear extracted protein, the SIE-containing oligonucleotide, and anti-phosphotyrosine^{705}STAT3. The mobility shift assay is shown in Fig. 2 *B*, *top*. Nuclear extracts from either unstimulated B-1 or B-2 cells or from B-2 cells treated with IL-6 for 15 min were incubated with the SIE oligonucleotide before EMSA. The SIF A complexes constitutively present in untreated B-1 cells and IL-6–treated B-2 cells were found to contain phosphotyrosine^{705}STAT3, as determined by immunoblotting material obtained from the native gel (Fig. 2 *B*, *bottom*). As a control, phosphotyrosine^{705}STAT3 electrophoresed in the absence of the SIE-containing oligonucleotide (no DNA control) was observed to migrate more slowly in the gel, distinguishing it from SIE-bound STAT3 (data not shown). These results confirm that STAT3 is constitutively present in B-1 nuclei, as indicated by the supershifting experiments outlined above and show that it is present as a tyrosine phosphorylated protein in the SIF A complex.

We further examined the phosphorylation status of STAT3 in nuclear extracts from B-1 and B-2 cells. CNTF has been reported to induce the tyrosine phosphorylation of two STAT3 isoforms, p88 and p89 (31). These isoforms, whose phosphorylation is also inducible by IL-6 (29, 32, 33), have been termed $STAT3_f$ (p88), for faster migrating and $STAT3_s$ (p89), for slower migrating (32, 34). Immunoblotting with antiserum specific for STAT3 phosphorylated on tyrosine^{705} showed that B-2 cells express little nuclear phosphotyrosine^{705}$STAT3_s$ or $STAT3_f$, as expected on the basis of EMSA analysis (Fig. 1 *A*). In comparison, B-1 cell

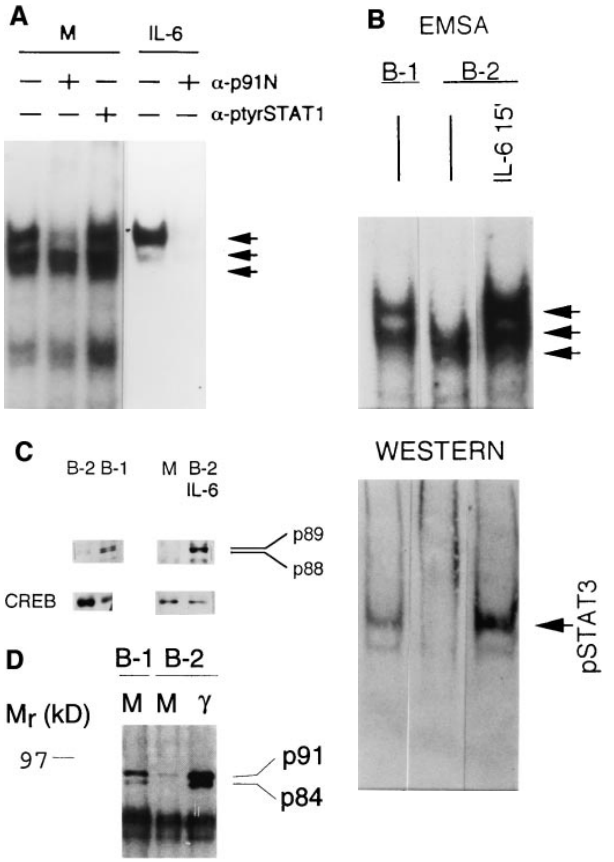

Figure 2. Immunoreactivity of B-1 cell SIF A with STAT3-specific antisera, and detection of constitutively expressed B-1 cell STAT 1 and 3 isoforms. (*A*) EMSA supershift/immunoinhibition analysis was performed using nuclear extracts from untreated B-1 cells (*M*) or B-2 cells stimulated with IL-6 and antibody to the NH_2-terminal region of STAT1, which has been shown to supershift STAT3 activated by IL-6 or CNTF (α-p91N). Antiserum specific for phosphorylated STAT1 (α-ptyrSTAT1) (26) was used as a control. Arrows indicate the positions of SIF A, B, and C. (*B*) B-1 cells were incubated in medium alone (−) and B-2 cells were either incubated in medium alone or were stimulated with IL-6 (1,000 U/ml) for 15 min, after which nuclear extracts were prepared. Nuclear extracted protein was analyzed by EMSA using the ^{32}P-labeled SIE-containing oligonucleotide probe and 5% nondenaturing PAGE. Retarded radiolabeled oligonucleotide was separated from DNA-binding proteins by a Shift–Western procedure, using electrotransfer to Whatman DE-81 paper and nitrocellulose filter paper, respectively. The SIE-containing oligonucleotide was visualized by autoradiography (*top*) and DNA-binding protein was immunoblotted with STAT3 phosphotyrosine^{705}-specific antibody and detected by ECL (*bottom*). The positions of the specific SIE-binding nucleoprotein complexes and immunoblotted phosphotyrosine^{705}STAT3 are indicated (*arrows*). (*C*) Immunoblot analysis of nuclear extracts from unstimulated B-2 or B-1 cells (*left*) or B-2 cells that were incubated with medium alone (*M*) or stimulated with IL-6 (B-2/IL-6; 1,000 U/ml for 15 min) (*right*), probed with antibody specific for STAT3 phosphorylated on tyrosine 705. Arrows indicate fast (p88) and slow (p89) migrating forms of STAT3. To test for equal loading of lanes, the blot was reprobed with an antibody to the constitutively expressed nuclear transcription factor, CREB (UBI, Lake Placid, NY). (*D*) Immunoblot analysis of nuclear extracts from untreated (M) B-1 or B-2 cells (lanes *1* and *2*) or B-2 cells stimulated with IFN-γ (5 ng/ml for 15 min; lane *3*), probed with antibody specific for phosphotyrosine^{701}STAT1 (26). Arrows indicate 91- and 84-kD isoforms of STAT1.

nuclear extracts were found to contain roughly equal levels of phosphotyrosine^{705}STAT3$_s$ and STAT3$_f$, respectively, which co-migrated with IL-6–induced phosphotyrosine^{705} STAT3 from B-2 cells (Fig. 2 *C*). In addition, immunoblotting untreated B-1 cell nuclear extracts with antibody specific for STAT1 phosphorylated on tyrosine^{701} (26) detected activated STAT1 of the p91 isoform, which was not present in nuclear extracts from untreated B-2 cells but was inducible by IFN-γ treatment (Fig. 2 *D*). Thus, the B-1–specific expression of SIF A correlates with the presence of the phosphotyrosine^{705} forms of STAT3$_s$ and STAT3$_f$ in nuclei from unstimulated B-1 cells, and B-1 cells also constitutively express a small amount of activated p91-STAT1.

The presence of SIF A and phosphotyrosine^{705}STAT3 in B-1 cells could not be due to macrophage contamination, because purified macrophages isolated by adherence during B cell purification from the same animals did not contain nuclear SIF A or phosphotyrosine^{705}STAT3, and histologic examination of B-1 populations revealed less than 2% macrophage contamination (data not shown). Further, overnight incubation of B-1 cells with neutralizing antibody to IL-10 before preparation of nuclear extracts did not result in diminution of the SIF A complex observed by EMSA (data not shown), suggesting that an IL-10 autocrine loop

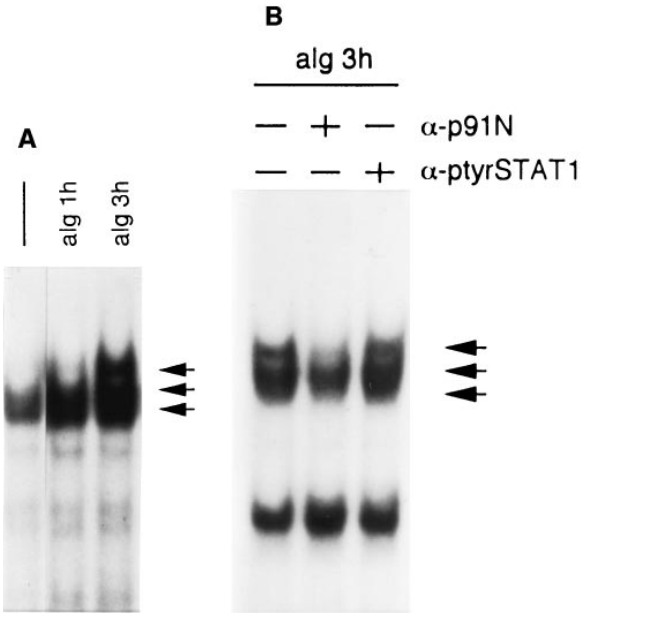

Figure 3. Inducible nuclear expression of STAT3 in B-2 cells treated with anti-Ig. (*A*) Delayed nuclear expression of SIF A in anti-Ig–stimulated B-2 cells. EMSA analysis for SIE-binding activity was carried out using nuclear extracts prepared from B-2 cells incubated in medium alone (−) or stimulated with F(ab′)$_2$ fragments of goat anti–mouse IgM (aIg; 15 μg/ml), as indicated. Arrows indicate positions of nucleoprotein complexes containing SIF A, B, and C. (*B*) Immunoreactivity of anti-Ig–induced B-2 cell SIF A with a STAT3-specific antiserum. Gel mobility supershift/immunoinhibition analysis was performed using nuclear extracts from B-2 cells stimulated with F(ab′)$_2$ GaMIgM (aIg) for 3 h and added to radiolabeled SIE-containing oligonucleotide before addition of antiserum specific for STAT3 (α-p91N) or for phosphorylated STAT1 (α-ptyrSTAT1); see legend to Fig. 1. Arrows indicate the position of nucleoprotein complexes containing SIF A, B, and C.

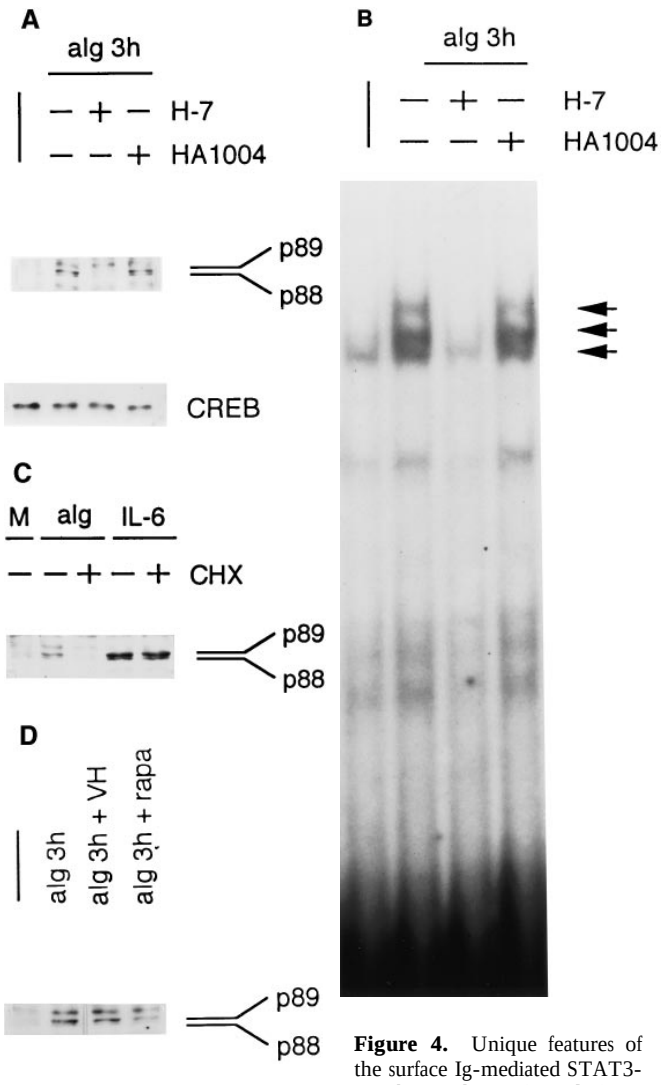

Figure 4. Unique features of the surface Ig-mediated STAT3-signaling pathway. (*A* and *B*) Induction of tyrosine phosphorylated STAT3 by anti-Ig in B-2 cells requires serine/threonine phosphorylation. Nuclear extracts were obtained from B-2 cells stimulated for 3 h with $F(ab')_2$ GaMIgM (aIg) alone or with aIg in the presence or absence of either the serine/threonine kinase inhibitor H-7, or its less active structural analogue, HA1004 (both at 25 μM; LC Laboratories) and probed with antibody specific for phosphotyrosine^{705}STAT3 (*A*). Arrows indicate fast (p88) and slow (p89) migrating forms of phosphorylated STAT3. To test for equal loading of lanes, the blot was reprobed with an antibody to the constitutively expressed nuclear transcription factor, CREB. Nuclear extracts were also analyzed by EMSA as described in Fig. 1 (*B*). Arrows indicate positions of nucleoprotein complexes containing SIF A, B, and C. (*C*) Induction of $STAT3_s$ after anti-Ig treatment is blocked by cycloheximide. Primary B cells were incubated in medium alone (M) or were treated with IL-6 (1,000 U/ml) for 15 min, or with $F(ab')_2$ goat anti–mouse IgM (15 μg/ml; aIg) for 3 h, after which nuclear extracts were prepared. Before stimulation, some B cell cultures were pretreated for 30 min with CHX (10 μg/ml) as indicated. Nuclear extracted protein was size separated by SDS-PAGE on 7.5% gels followed by immunoblotting with phosphotyrosine^{705}STAT3–specific antibody, detected by ECL. Arrows indicate fast (p88) and slow (p89) migrating forms of phosphorylated STAT3. (*D* and *E*) Rapamycin inhibits anti-Ig–induced activation of STAT3. Primary B cells (1.5×10^7) were incubated in medium alone (−) or were stimulated with $F(ab')_2$ goat anti–mouse IgM (15 μg/ml; aIg) for the indicated times, after which nuclear extracts were prepared. Before stimulation, some B cell cultures were pretreated for 15 min with either rapamycin at 20 ng/ml (rapa) or ethanol (vehicle, VH). Nuclear extracted protein was size separated by SDS-PAGE on 7.5% gels followed by immunoblotting with phosphotyrosine^{705}STAT3–specific antibody, detected by ECL (*D*). The positions of molecular size markers and phospho-STAT3 (*arrow*) are indicated. Nuclear extracts were also analyzed by EMSA as described in Fig. 1 (*E*). Arrows indicate positions of nucleoprotein complexes containing SIF A, B, and C.

does not account for the observed elevated levels of STAT3 in unstimulated B-1 cells and that constitutive STAT3 expression is intrinsic to this population.

Previous work has shown that anti-Ig treatment of B-2 cells results in STAT1 activation (35). It has been suggested that B-1 cells represent a population of conventional B cells previously activated through their antigen receptors. For these reasons, STAT3 activation was evaluated in nuclear extracts from B-2 cells treated with anti-Ig. Nuclear extracts from anti-Ig–treated B-2 cells formed a SIF A complex with the SIE similar to that observed in untreated B-1 cells, although this activity was only present in extracts from cells stimulated for 3 h or more (Fig. 3 *A*). As with B-1 SIF A, anti-p91N but not antiserum to phosphotyrosine^{701}STAT1 disrupted SIF A induced by anti-Ig in B-2 cells (Fig. 3 *B*) and formed a supershifted complex with SIF A that was visible in longer exposures and co-migrated with a similar complex recognized by anti-p91N in IL-6–treated B cells (data not shown). Further, phosphotyrosine$^{705}STAT3_s$ was detected by immunoblotting using nuclear extracts from B-2 cells treated for 3 h with anti-Ig, although little phosphotyrosine$^{705}STAT3_f$ was observed (Fig. 4 *A*). B cells stimulated with anti-Ig for less than 3 h did not contain nuclear phosphotyrosine$^{705}STAT3_s$ or $STAT3_f$ (data not shown). These results suggest that cross-linking sIg in B-2 cells generates activated nuclear STAT3 of predominantly the $STAT3_s$ isoform, much like IL-6 treatment of B-2 cells but dissimilar from the phosphotyrosine^{705}STAT3 profile of unstimulated B-1 cells, in which levels of $STAT3_s$ and $STAT3_f$ are nearly equal (Fig. 2 *C*). Thus, constitutively expressed B-1 cell STAT3 is not the same as STAT3 induced in B-2 cells by sIg or cytokine receptor engagement.

The formation and transcriptional activity of cytokine-induced STAT3 complexes have been shown to require inducible serine phosphorylation (32, 36). Because many sIg triggered downstream events are PKC dependent, we tested whether anti-Ig induction of STAT3 in B-2 cells is sensitive to inhibition of serine/threonine phosphorylation, using the inhibitor, H7. The induction of phosphotyrosine$^{705}STAT3_s$ by anti-Ig was completely inhibited by H7 but not by treatment with (the control analog) HA1004 (Fig. 4 *A*). Preincubation with H7 but not with HA1004

also completely blocked the formation of the SIF A–binding complex in EMSA experiments conducted using nuclear extracts from B-2 cells stimulated with anti-Ig for 3 h (Fig. 4 *B*). These results suggest that nuclear localization of phosphotyrosine^{705}STAT3$_s$ and the appearance of nuclear SIF A in B-2 cells stimulated by anti-Ig requires serine/threonine phosphorylation and further implicate STAT3$_s$ in the composition of the anti-Ig–induced SIF A complex.

The delayed tyrosine phosphorylation of STAT3 after sIg ligation in B-2 cells suggested that the synthesis of an intermediary protein is required for this response. To test this possibility, B-2 cells were stimulated with anti-Ig in the presence of the protein synthesis inhibitor cycloheximide (CHX) and nuclear extracts were prepared. CHX completely blocked the induction of phosphotyrosine^{705}STAT3$_s$ in nuclear extracts from B-2 cells treated with anti-Ig for 3 h, whereas CHX had no effect on phosphotyrosine^{705}STAT3 stimulated by IL-6 (Fig. 4 *C*). CHX also abrogated the formation of the SIF A–binding complex in EMSA experiments performed using nuclear extracts from B-2 cells stimulated with anti-Ig for 3 h (data not shown). Thus, de novo protein synthesis is required for induction of both SIF A and of phosphotyrosine^{705}STAT3$_s$ by anti-Ig.

Since anti-Ig is a mitogenic stimulus for B-2 cells, we reasoned that induction of STAT3 via this novel mechanism may be sensitive to immunosuppressive drugs that inhibit B cell proliferation, such as cyclosporin A, FK506, and rapamycin (37, 38). Immunoblot analysis of nuclear extracted protein showed substantial inhibition of anti-Ig–induced phosphotyrosine^{705}STAT3$_s$ by rapamycin (Fig. 4 *D*). Rapamycin also significantly blocked the formation of the anti-Ig–inducible SIF A complex (Fig. 4 *E*). This effect of rapamycin is specific for sIg-triggered STAT3 because induction of phosphotyrosine^{705}STAT3 by IL-6 was not affected by rapamycin (data not shown). Further, B-2 cell treatment with CsA had a minimal effect on nuclear expression of phosphotyrosine^{705}STAT3$_s$ after anti-Ig stimulation but completely inhibited nuclear phosphotyrosine^{705}STAT3$_s$ induced by the combination of PMA and the calcium ionophore, ionomycin (data not shown) demonstrating an additional level of specificity for the effect of rapamycin on anti-Ig–induced STAT3.

Both the delayed appearance and dependence on protein synthesis of phosphotyrosine^{705}STAT3 in B-2 cells after anti-Ig stimulation raised the possibility that sIg-mediated STAT3 induction may be due to the release of cytokines from the B cells themselves or from other contaminating cells in the B-2 cell preparation after treatment with anti-Ig. To address this question, nuclear extracts from the mature B cell line BAL-17 were prepared and immunoblotted for phosphotyrosine^{705}STAT3 after stimulation with anti-Ig. Phosphotyrosine^{705}STAT3$_s$ was induced in BAL-17 B cells by anti-Ig treatment with similar kinetics to that observed in B-2 cells (data not shown) ruling out a role for a factor secreted by a contaminating non–B cell. In addition, culture supernatants from B cells stimulated by anti-Ig for 3 h were transfered to naive cells, from which nuclear extracts were prepared after 15 min and tested for the presence of phosphotyrosine^{705}STAT3 by immunoblotting. Supernatants from cultures treated with anti-Ig for 3 h did not induce appreciable rapid tyrosine phosphorylation of STAT3 in previously naive cells (data not shown), as would be expected of a cytokine-mediated response. These results, coupled with the sensitivity of this response to rapamycin, which does not inhibit cytokine-mediated STAT signaling, suggest that the delayed tyrosine phosphorylation of STAT3 is specific to anti-Ig treatment and is not the result of cytokine release or synthesis triggered by cell activation.

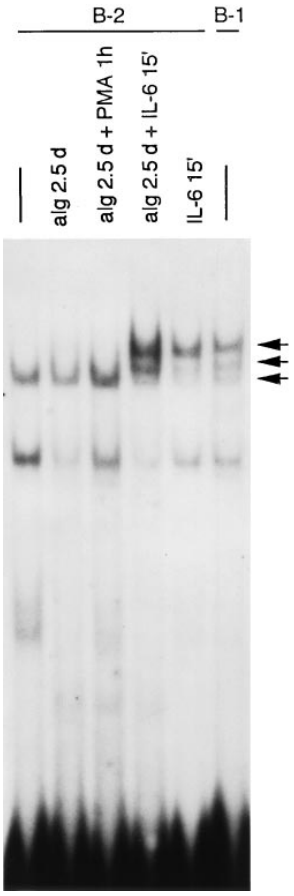

Figure 5. Failure of prolonged anti-Ig treatment of B-2 cells to reproduce the B-1 cell nuclear STAT3 profile. Nuclear extracts were prepared from untreated B-1 cells and from B-2 cells incubated with either medium alone (−), or treated with IL-6 for 15 min; nuclear extracts were also prepared from B-2 cells treated with anti-Ig (2.5 μg/ml) for 2.5 d or 2.5 d anti-Ig–treated B-2 cells subsequently stimulated with either PMA for 1 h or IL-6 for 15 min, as indicated. EMSA was performed as described in Fig. 1. Arrows indicate positions of nucleoprotein complexes containing SIF A, B, and C.

Prolonged exposure of B-2 cells to anti-Ig (e.g., for 2.5 d) has been shown to result in the acquisition of surface CD5 expression and proliferative responsiveness to PMA (39, 40). The possibility that prolonged sIg crosslinking produces a B-1–like basal level of nuclear activated STAT3 was tested by treating B-2 cells with anti-Ig for several days before nuclear extraction. Although B-2 cells treated with anti-Ig for 2.5 d responded to PMA by cell cycle progression to S phase, sIg-mediated nuclear SIF A (which was apparent at 3 h) had disappeared by this time (Fig. 5). This result indicates that STAT3 induced by anti-Ig in B-2 cells is only transiently expressed, and thus long term T cell–independent type II (TI-2) antigenic stimulation of B-2 cells does not recapitulate the profile of activated STAT3 characteristic of B-1 cells, despite inducing other B-1–like changes. These results suggest that activated STAT3 expression is an intrinsic and unique characteristic of B-1 cells.

In conclusion, we have identified constitutive nuclear activated STAT3 in normal murine B-1 lymphocytes, representing the first nuclear transcriptional identifier for this developmentally regulated B cell population. The B-1 cell subset has been linked to spontaneously arising B cell tumors, and STAT3 has been found to be activated in *v-abl*-transformed B cells, HTLV-I–transformed T cells, and *v-src*–transformed fibroblasts (17–20). Basal levels of nuclear phosphorylated STAT3 may reflect, or may cause, the activated state of B-1 cells, and may contribute to the self-renewing growth characteristics and the oncogenic potential of normal B-1 cells in vivo. In contrast, both *egr-1* and c-*myc* mRNA levels do not differ between B-1 and B-2 cells (41).

There has been considerable debate over whether B-1 cells are derived from a separate lineage of progenitor cells or represent B-2 cells that have undergone internal biochemical and external cell surface marker changes due to prior activational or differentiative responses, such as those delivered by TI-2 antigens (42, 43). Our data suggest that one activity of sIg cross-linking in conventional B cells is to activate STAT3, which occurs in delayed fashion, involves phosphorylation of tyrosine^{705}, and is dependent upon de novo protein synthesis, serine/threonine phosphorylation, and the participation of a rapamycin-inhibitable kinase. Thus, the B cell antigen receptor is coupled to nuclear expression of activated STAT proteins (this work and references 16, 35). Notably, many features of this coupling stand in stark contrast to the accepted paradigm for STAT activation mediated by cytokine receptors, in which STAT phosphorylation occurs rapidly, does not require protein synthesis, and is independent of rapamycin-sensitive kinase activity. However, the basal presence of both activated $STAT3_s$ and $STAT3_f$ in unstimulated B-1 cells contrasts with the transient induction of predominantly the $STAT3_s$ isoform in anti-Ig stimulated B-2 cells and suggests that cross-linking sIg alone does not result in similar nuclear expression, in B-2 cells, of this activated transcription factor present in B-1 cells. Therefore, constitutive B-1 cell STAT3 expression suggests that the development of these B cells cannot be explained by TI-2 antigen–mediated influences alone, and that STAT proteins play a role in directing the unique behavioral and phenotypic characteristics of this population of normal cells.

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