Differential Intrinsic Enzymatic Activity of Syk and Zap-70 Protein-tyrosine Kinases*

(Received for publication, April 17, 1996, and in revised form, June 18, 1996)

Sylvain Latour‡‡§§, Lionel M. L. Chow¶¶, and André Veillette¶¶***‡‡‡‡‡‡§§§§

From the ‡‡McGill Cancer Centre and Departments of ‡‡Biochemistry, **‡‡Medicine, and ‡‡‡‡Oncology, McGill University, Montréal, Canada H3G 1Y6 and the §§Departments of Medicine and Oncology, Montreal General Hospital, Montréal, Canada H3G 1A4

Syk and Zap-70 are related protein-tyrosine kinases implicated in antigen and Fc receptor signaling. While Zap-70 is restricted to T-cells and natural killer cells, Syk accumulates in B-cells, mast cells, platelets, and immature T-cells. In addition, we found that an isoform of Syk (SykB), which carries a 23-amino acid deletion in the “linker” region, is prominently expressed in bone marrow. To better understand the relative impact of Syk, SykB, and Zap-70 on signal transduction, we compared their intrinsic enzymatic properties in transiently transfected COS-1 cells and in hemopoietic cells. Using modified versions of these enzymes bearing a common Myc epitope at the amino terminus, we determined that the ability of Syk and SykB to undergo autophosphorylation and to phosphorylate erythrocyte band 3 in immune complex kinase reactions was at least 100-fold greater than that of Zap-70. Similarly, Syk and SykB, but not Zap-70, caused prominent tyrosine phosphorylation of p120c-catenin in COS-1 cells. A similar pattern of activity was also noted for endogenous Syk and Zap-70 from hemopoietic cells. To understand the structural basis for these characteristics, we also created and analyzed a series of chimeras between Syk and Zap-70. These studies indicated that the catalytic domain of Syk and Zap-70, but not their SH2 domains, linker region or carboxyl-terminal tail, was responsible for their respective activity. Taken together, these data demonstrated that the intrinsic enzymatic activity of Syk and SykB is superior to that of Zap-70 and that such a distinction relates to structural variations in the catalytic domain.

The Syk family of nonreceptor protein-tyrosine kinases (PTKs)† comprises two known members termed Zap-70 and Syk (reviewed in Ref. 1). Zap-70 is solely expressed in T-cells and natural killer (NK) cells, whereas Syk accumulates in most hemopoietic cell types, including B-cells, mast cells, platelets, and immature T-cells. The structure of Syk family kinases is highly conserved, including from the amino terminus to the carboxyl terminus: 1) two Src homology 2 (SH2) domains, implicated in interactions with tyrosine-phosphorylated proteins; 2) a “linker” region of 80–110 amino acids, which is postulated to bind cellular effectors (2); 3) a catalytic domain, including sites of ATP binding and autophosphorylation; and 4) a short carboxyl-terminal extension of yet undetermined function (Fig. 1A).

Over the past few years, evidence has accumulated that Zap-70 and Syk participate in signal transduction through antigen and Fc receptors in hemopoietic cells (reviewed in Ref. 1). Engagement of these receptors by the appropriate ligand triggers tyrosine phosphorylation of receptor-associated subunits, within a tyrosine-based motif termed ITAM (for immunoreceptor tyrosine-based activation motif). This event is seemingly mediated by members of the Src family of PTKs. Subsequently, Zap-70 and Syk bind doubly phosphorylated ITAMs via their tandem SH2 domains and undergo phosphorylation on multiple tyrosine residues. By reconstituting these interactions in COS cells, it has been concluded that tyrosine phosphorylation of Zap-70 and Syk is mediated both by Src family kinases and, to a lesser extent, by autophosphorylation (3–6). Binding of Zap-70 and Syk to ITAMs also augments their catalytic activity, presumably as a result of tyrosine phosphorylation and conformational modification (7–10). Tyrosine phosphorylation of Syk family kinases may also create docking sites allowing the recruitment of other SH2 domain-containing signaling molecules (2, 10).

Genetic evidence supports the notion that Zap-70 and Syk provide essential functions in signaling through antigen and Fc receptors. Takata et al. (11) showed that Syk-deficient DT-40 B-cells exhibited markedly decreased B-cell antigen receptor (BCR)-induced protein tyrosine phosphorylation and calcium flux. Similarly, expression of kinase-defective versions of Syk blocked signaling through Fc receptor for IgE (FcεR1) in RBL-2H3 basophilic leukemia cells (12), whereas introduction of Zap-70 polypeptides devoid of catalytic domain inhibited T-cell antigen receptor-mediated signals in Jurkat T-cells (13). A crucial role for Zap-70 and Syk in hemopoietic cell development was also implied by analyses of human or mouse mutants lacking these enzymes. Humans and mice lacking Zap-70 were shown to have markedly abnormal T-cell maturation (14–17). Conversely, Syk-deficient mice demonstrated altered B-cell development, reduced hemostasis, and embryonic lethality (18, 19). Proper maturation of some subsets of γδ T-cells was also defective in these animals.

Even though the exact mechanism by which Zap-70 and Syk mediate their biological functions is not understood, the creation of chimeric molecules linking Zap-70 or Syk to the transmembrane and extracellular domains of unrelated molecules

---

* This work was supported by grants from the Cancer Research Society Inc., the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Leukemia Research Fund of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a Fellowship from the Leukemia Research Fund of Canada.

¶ Supported by a Steve Fonyo Studentship from the National Cancer Institute of Canada.

** Scientist of the Medical Research Council of Canada. To whom correspondence should be addressed: Rm. 715, McIntyre Medical Sciences Bldg., McGill University, 3655 Drummond St., Montréal, Canada H3G 1Y6. Tel.: 514-398-8936; Fax: 514-398-6769; E-mail: veillette@medcor.mcgill.ca.

† The abbreviations used are: PTK, protein-tyrosine kinase; mAb, monoclonal antibody; ITAM, immunoreceptor tyrosine-based activation motif; cdb3, cytoplasmic domain of erythrocyte band 3.
indicated that they directly couple to effectors of cellular activation (20, 21). Antibody-mediated aggregation of a CD16-CD7-Syk chimera was sufficient to cause an elevation of cytoplasmic calcium and induce cytolytic functions in T-cell lines (20). Similarly, it provoked degranulation of RBL-2H3 cells (21). Intriguingly though, stimulation of the equivalent Zap-70 chimera induced a rise in intracellular calcium in T-cells, but failed to induce cytolysis (20). Co-aggregation of this chimera with a Fyn or Lck chimera was necessary to trigger cytotoxicity. These data led to the proposal that the function and/or regulation of Zap-70 and Syk may not be identical.

To evaluate whether these findings were caused by differences in the intrinsic PTK activity of Zap-70 and Syk, we undertook a series of experiments contrasting their biochemical properties. Using versions of mouse Zap-70 and Syk carrying a common Myc epitope at their amino terminus, we found that Syk is significantly more active than Zap-70 both in vitro and in vivo assays. Such a difference was observed not only for moloney murine leukemia virus gag but also for endogenous Zap-70 and Syk polypeptides contained in hemopoietic cells. Through construction of chimeras between Zap-70 and Syk, it was also established that these biochemical differences relate to sequence variations in the catalytic domain of these polypeptides. Collectively, these data implied that the ability of Syk to participate in hemopoietic cell signaling is greater than that of Zap-70 at least in part as a consequence of its superior intrinsic PTK activity.

MATERIALS AND METHODS

Cells—COS-1 cells were propagated in a minimal essential medium supplemented with 10% fetal calf serum and antibiotics. WEHI-231 mouse B-lymphoma cells were obtained from the American Type Culture Collection, Rockville, MD. A202/3 is a mouse B-cell lymphoma cell line (22), and BI-141 is an antigen-specific mouse T-cell hybridoma (23). All hemopoietic cells were grown in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. Splenocytes, thymocytes, and bone marrow cells were isolated from 4–6-week-old Balb/c mice. For preparation of splenocytes and bone marrow cells, erythrocytes were depleted by incubation in ACK buffer (24).

Antibodies—Anti-Syk antibodies were produced in rabbits using a Tryptophan protein encompassing amino acids 258–356 of mouse Syk (25, 26). Rabbit antisera directed against amino acids 253–259 of Zap-70, or against c-Cbl, were described elsewhere (27, 28). Monoclonal antibody (mAb) 9E10 is a mouse antibody directed against human c-Myc (29). mAb 4G10 is a mouse anti-phosphotyrosine mAb purchased from Upstate Biotechnology Inc., Lake Placid, New York. Affinity-purified rabbit anti-phosphotyrosine antibodies were reported previously (30).

cDNAs—Mouse syk cDNAs were cloned from a day 16 fetal thymus cDNA library (kindly provided by Dr. Louis Matis, Alexion Pharmaceuticals, New Haven, CT), using a partial cDNA corresponding to the linker region of Syk as a probe. Representative full-length cDNAs were sequenced to ensure that they contained no mutation (data not shown). cDNAs were isolated in the multiple cloning site of pBluescript Myc tag (from Dr. A. Shaw). As a result, the sequence MASMOKLI SEED1NGNS was added to the amino terminus of Syk, SykB, and Zap-70, thereby creating Myc-Syk, Myc-SykB, and Myc-Zap-70. The underlined amino acids are derived from human Myc and are recognized by mAb 9E10 (Ref. 29).

Generation of Syk-Zap and Syk-Syb Chimeras—Chimeras between Syk and Zap-70 were produced by polymerase chain reaction (Fig. 7). Syk-Zap contained the SH2 domains and linker region of Syk (amino acids 1–364; Ref. 26), fused to the carboxyl-terminal tail of Zap-70 (amino acids 337–618; Ref. 31). Zap-Syk encompassed the SH2 domains and linker region of Zap-70 (amino acids 1–336) and the kinase and carboxyl-terminal tail of Syk (amino acids 365–629). Zap-Syk contained the SH2 tail contained the SH2 domains, linker region, and kinase region of Syk (amino acids 1–620), linked to the carboxyl-terminal tail of Zap-70 (amino acids 593–618). Finally, Zap/Syk-tail possessed the SH2 domains, linker region and kinase domain of Zap-70 (amino acids 1–592), fused to the carboxyl-terminal tail of Syk (amino acids 621–629). The nucleotide sequence of all chimeric cDNAs was verified by sequencing (data not shown). All chimeric proteins also contained a Myc epitope at their amino terminus.

Transfections—cDNAs were individually inserted in the multiple cloning site of pXM139, a vector that bears the SV40 origin of replication. COS-1 cells were transfected by the DEAE-dextran method, using a fixed total amount of DNA (28). After 12 h, cells were incubated with chloroquine (60 μg/ml) for 5 h. Following an additional growth period of 48 h, cells were processed for immunoprecipitation and immunoblotting.

Immunoprecipitations and Immunoblots—After washing in phosphate-buffered saline, cells were lysed in TNE buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 2 mM EDTA) supplemented with 10 μg/ml each of the protease inhibitors leupeptin, aprotinin, N-tosyl-L-phenylalnine chloromethylketone, N-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Polypeptides were recovered by immunoprecipitation using the indicated antibodies. Immune complexes were collected with Staphylococcus aureus protein A (Pansorbin, Calbiochem), coupled, if indicated, to rabbit anti-mouse immunoglobulin IgG. Immunoprecipitates were washed three times with TNE buffer containing 1 mM sodium orthovanadate. Proteins were then eluted in sample buffer, boiled, electrophoresed in 8% SDS-polyacrylamide gel electrophoresis gels, and transferred onto Immobilon membranes (Millipore, Mississauga, Ontario, Canada) for immunoblotting. Immunoblots were performed according to a previously described protocol (33). After incubation with 125I-protein A (Amersham Canada), membranes (Millipore, Mississauga, Ontario, Canada) were incubated with the antibodies, and the membranes were washed with TNE buffer containing 1 mM sodium orthovanadate. Proteins were then visualized with phosphorimager. In deploration experiments, phosphotyrosine-containing proteins were removed by immunoprecipitation with anti-phosphotyrosine mAb 4G10. Then, Myc-tagged polypeptides were isolated by immunoprecipitation with mAb 9E10 and tested as described in the text.

Immune Complex Kinase Assays—These assays were done under linear conditions according to a previously described protocol (34), with the exception that the kinase reactions were performed in 25 μl of a buffer containing 20 mM Tris, pH 7.5, 10 mM MnCl2, 10 mM MgCl2, 1 μM nonradioactive ATP, and 12.5 μCi of [γ-32P]ATP (3000 Ci/mmol; DuPont NEN), as well as 1 μg of the cytoplasmic domain of erythrocyte band 3 (c3β). Reactions were conducted at room temperature for the indicated periods of time. Data were quantitated with a phosphorimager.

RESULTS

Expression of an Additional Syk Isoform in Normal Mouse Bone Marrow Cells—Previous studies have identified an alternative syk transcript in human Jurkat leukemia T-cells (35) and RBL-2H3 rat basophilic leukemia cells (36). This variant, termed sykB, carried a deletion of a 69-nucleotide exon (36) and was expected to encode a Syk protein lacking 23 amino acids in the linker region (amino acids 279–292) for the mouse Syk protein; Ref. 26). While attempting to clone syk cDNAs from normal mouse bone marrow cells, we found one of 10 independent cDNA clones having a similar 69-nucleotide deletion (Fig. 1; data not shown). To ascertain whether transcripts corresponding to this cDNA accumulated in mouse cells, ribonuclease

 Downloaded from http://www.jbc.org/ by guest on January 24, 2018
protection assays were performed using total RNAs from a series of cell lines and tissues (Fig. 2A). While regular syk transcripts (hereafter identified as syk) were expected to protect a 239-nucleotide fragment of the riboprobe, sykB RNAs were predicted to cover a smaller 175-nucleotide fragment.

syk RNAs were easily detected in fetal and adult thymus (Fig. 2A, lanes 1 and 2), spleen (lane 3), normal bone marrow (lane 4), as well as in the B-cell lines WEHI-231 (lane 8) and A20/2J (lane 9), and the mast cell line MC-9 (lane 11). Smaller amounts were observed in the mastocytoma cell line P815 (lane 10). In contrast, brain tissue (lane 5), BI-141 T-cells (lane 6), and EL-4 T-cells (lane 7) did not contain any appreciable quantity of syk. syk-expressing tissues and cell lines also frequently contained limited amounts (approximately 10% of total) of transcripts that protected a 175-nucleotide fragment and presumably corresponded to sykB RNAs. One notable exception was normal bone marrow (lane 4), in which roughly 50% of all syk RNAs protected the 175-nucleotide fragment.

To ascertain whether the SykB protein was expressed in normal cells, Syk polypeptides were immunoprecipitated from spleen, thymus, or bone marrow and were detected by immunoblotting with a polyclonal rabbit anti-Syk serum (Fig. 2B). This assay revealed that spleen (lane 1) and thymus (lane 2) primarily contained a 72-kDa immunoreactive product (p72syk), which comigrated with the Syk protein immunoprecipitated from COS-1 cells transfected with a mouse syk cDNA (lane 5). The Syk protein could not be clearly identified in these cells. In contrast, normal bone marrow cells (lane 3) possessed equal amounts of two distinct proteins, resolving at 70 and 72 kDa in these gels. These polypeptides comigrated with the Syk proteins recovered from COS-1 cells transfected with sykB and syk cDNAs, respectively (lanes 4 and 5). Whereas spleen and bone marrow expressed approximately equivalent quantities of total syk transcripts (Fig. 2A), it is noteworthy that bone marrow cells contained significantly lower amounts of Syk proteins (Fig. 2B). Possibly, the Syk proteins were less stable in bone marrow cells. Alternatively, the translational efficiency of syk RNAs was perhaps diminished in these cells.

Syk and SykB, but Not Zap-70, Enhance Protein-tyrosine Phosphorylation in COS-1 Cells—In order to adequately compare the biochemical activity of Syk, SykB, and Zap-70,
epitope-tagged versions of these enzymes were engineered. A sequence from the human c-Myc protein (SMEQKLISEED-LNN) was linked to the amino terminus of the three kinases, as outlined under “Materials and Methods.” This “tag” can be recognized with anti-Myc mAb 9E10 (Ref. 29). The resulting proteins, termed Myc-Syk, Myc-SykB, and Myc-Zap-70 were expressed in COS-1 cells by transient transfection. Immunoblotting of total cell lysates with mAb 9E10 demonstrated that all Myc-tagged proteins were expressed in equivalent amounts (data not shown; Fig. 3B, bottom panel). To begin characterizing the properties of these polypeptides, their extent of tyrosine phosphorylation was directly examined. Lysates were immunoprecipitated with anti-Myc mAb 9E10 and subjected to immunoblotting with anti-phosphotyrosine antibodies (Fig. 3A, top panel). Myc-Syk (lane 2) and Myc-SykB (lane 3) were clearly tyrosine-phosphorylated in COS-1 cells. In contrast, however, Myc-Zap-70 (lane 4) did not contain any appreciable amount of phosphotyrosine. These differences did not relate to variations in the abundance of the three polypeptides, as judged by an immunoblot of parallel immunoprecipitates with anti-Myc mAb 9E10 (Fig. 3A, bottom panel). Similar results were obtained with nontagged versions of these enzymes (data not shown).

Recently, the proto-oncogene product p120c-cbl was shown to undergo prominent tyrosine phosphorylation during antigen receptor-mediated activation of T-cells and B-cells (37, 38). This phosphorylation is thought to be mediated both by Src-related enzymes and Syk-related enzymes. Hence, to further compare the activity of Syk, SykB, and Zap-70, we examined their capacity to phosphorylate c-Cbl in COS-1 cells. Cells were transfected with cDNAs coding for the various Syk-related enzymes as described above, in the absence or presence of a mouse c-cbl cDNA. The extent of tyrosine phosphorylation of p120c-cbl was determined by immunoblotting anti-Cbl immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 3B, top panel). We found that expression of Myc-Syk (lane 6) and Myc-SykB (lane 7), but not of Myc-Zap-70 (lane 8), provoked tyrosine phosphorylation of the 120-kDa c-Cbl protein.

To ensure that the greater ability of Myc-Syk to phosphorylate c-Cbl was not the result of functional modifications provoked by addition of the Myc epitope, we also determined the effects of wild-type Syk and Zap-70 on c-Cbl tyrosine phosphorylation (Fig. 3C). This assay revealed that, like Myc-Syk (lane 3), Syk (lane 2) stimulated tyrosine phosphorylation of c-Cbl in COS-1 cells. Furthermore, as was the case for Myc-Zap-70 (lane 5), Zap-70 (lane 4) failed to induce any detectable tyrosine phosphorylation of the substrate.

Syk and SykB Have Greater Enzymatic Activity than Zap-70

in in Vitro Assays—The activity of Syk, SykB, and Zap-70 was also measured in in vitro immune complex kinase assays. Transfected COS-1 cells were lysed in Nonidet P-40-containing buffer, and Myc-tagged proteins were recovered by immunoprecipitation with anti-Myc mAb 9E10. After several washes, immune complexes were incubated for various periods of time at room temperature, in the presence of [γ-32P]ATP, nonradioactive ATP, MnCl₂, and MgCl₂, as well as the exogenous substrate erythrocyte band 3 (cbl3; Ref. 39). The phosphorylated products of these reactions were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography (Fig. 4, top panel). The relative abundance of the Myc-tagged enzymes, as well as of heavy chain of Ig, are indicated on the left; whereas those of prestained molecular weight markers are shown on the right. Exposures: top panel, 12 h; bottom panel, 4 h. B and C, COS-1 cells were also transfected with the indicated cDNAs, in the absence or presence of a mouse c-cbl cDNA. c-Cbl was recovered by immunoprecipitation from 250 μg of cellular proteins and probed by immunoblotting with anti-phosphotyrosine antibodies. The abundance of c-Cbl, and of the various Syk-related enzymes, was determined by immunoblotting of equivalent amounts (30 μg) of cell lysates with the indicated antibodies. B, comparison of Myc-Syk, Myc-SykB, and Myc-Zap-70. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 24 h; middle panel, 8 h; bottom panel, 2 h. C, comparison of Myc-tagged and nontagged versions of Syk and Zap-70. All cells were transfected with the c-cbl cDNA. For the bottom panel, lanes 1–3 were immunoblotted with anti-Syk antibodies, whereas lanes 4 and 5 were reacted with anti-Zap-70 antibodies. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 8 h; middle panel, 8 h; bottom panel, 8 h.

Fig. 3. Impact of Syk family kinases on intracellular protein tyrosine phosphorylation in COS-1 cells. COS-1 cells were transiently transfected with the indicated cDNAs. Equivalent amounts of lysates (250 μg) were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. A, tyrosine phosphorylation of Myc-tagged proteins. Myc-tagged proteins were immunoprecipitated with mAb 9E10 and immunoblotted with rabbit anti-phosphotyrosine antibodies (top panel) or anti-Myc antibodies (bottom panel). The migrations of Myc-Syk and Myc-SykB, as well as of heavy chain of Ig, are indicated on the left, whereas those of prestained molecular weight markers are shown on the right. Exposures: top panel, 12 h; bottom panel, 4 h. B and C, COS-1 cells were also transfected with the indicated cDNAs, in the absence or presence of a mouse c-cbl cDNA. c-Cbl was recovered by immunoprecipitation from 250 μg of cellular proteins and probed by immunoblotting with anti-phosphotyrosine antibodies. The abundance of c-Cbl, and of the various Syk-related enzymes, was determined by immunoblotting of equivalent amounts (30 μg) of cell lysates with the indicated antibodies. B, comparison of Myc-Syk, Myc-SykB, and Myc-Zap-70. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 24 h; middle panel, 8 h; bottom panel, 2 h. C, comparison of Myc-tagged and nontagged versions of Syk and Zap-70. All cells were transfected with the c-cbl cDNA. For the bottom panel, lanes 1–3 were immunoblotted with anti-Syk antibodies, whereas lanes 4 and 5 were reacted with anti-Zap-70 antibodies. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 8 h; middle panel, 8 h; bottom panel, 8 h.

FIG. 3. Impact of Syk family kinases on intracellular protein tyrosine phosphorylation in COS-1 cells. COS-1 cells were transiently transfected with the indicated cDNAs. Equivalent amounts of lysates (250 μg) were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. A, tyrosine phosphorylation of Myc-tagged proteins. Myc-tagged proteins were immunoprecipitated with mAb 9E10 and immunoblotted with rabbit anti-phosphotyrosine antibodies (top panel) or anti-Myc antibodies (bottom panel). The migrations of Myc-Syk and Myc-SykB, as well as of heavy chain of Ig, are indicated on the left, whereas those of prestained molecular weight markers are shown on the right. Exposures: top panel, 12 h; bottom panel, 4 h. B and C, COS-1 cells were also transfected with the indicated cDNAs, in the absence or presence of a mouse c-cbl cDNA. c-Cbl was recovered by immunoprecipitation from 250 μg of cellular proteins and probed by immunoblotting with anti-phosphotyrosine antibodies. The abundance of c-Cbl, and of the various Syk-related enzymes, was determined by immunoblotting of equivalent amounts (30 μg) of cell lysates with the indicated antibodies. B, comparison of Myc-Syk, Myc-SykB, and Myc-Zap-70. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 24 h; middle panel, 8 h; bottom panel, 2 h. C, comparison of Myc-tagged and nontagged versions of Syk and Zap-70. All cells were transfected with the c-cbl cDNA. For the bottom panel, lanes 1–3 were immunoblotted with anti-Syk antibodies, whereas lanes 4 and 5 were reacted with anti-Zap-70 antibodies. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular weight markers are shown on the right. Exposures: top panel, 8 h; middle panel, 8 h; bottom panel, 8 h.
enzymatic activities of Syk and Zap-70

Proteins was also monitored by anti-Myc immunoblotting of parallel immunoprecipitates subjected to kinase reactions in the absence of radioactive ATP (bottom panel). This experiment demonstrated that Myc-Syk (lanes 6–12) and Myc-SykB (lanes 13–18) underwent rapid phosphorylation in vitro. Moreover, they caused marked phosphorylation of erythrocyte band 3. In comparison, Myc-Zap-70 (lanes 1–6) was weakly phosphorylated and induced only minimal phosphorylation of the exogenous substrate. Quantitation of these data suggested that the kinase activity of Myc-Syk and Myc-SykB was at least 100-fold greater than that of Myc-Zap-70 in vitro. Similar results were obtained when lower amounts of Myc-tagged proteins were used or when the kinase reactions were conducted for shorter periods of time, under assay conditions that we had demonstrated to be linear (data not shown). Moreover, analogous findings were made over a wide range of reaction conditions, which included variations in buffer, pH, co-factor, and temperature (data not shown).

To ensure that the greater activity of Myc-Syk and Myc-SykB in these assays was not due to their prior tyrosine phosphorylation in COS-1 cells, tyrosine-phosphorylated molecules were depleted from cell lysates, and immune complex kinase reactions were performed on the remaining Myc-tagged molecules (Fig. 5). Even though all tyrosine-phosphorylated Myc-Syk (Fig. 5A, top panel, lane 4) and Myc-SykB (lane 5) could be removed in this manner, these two enzymes (Fig. 5B, lanes 4 and 5, respectively) remained more efficient than Myc-Zap-70 (lane 6) at autophosphorylation and phosphorylation of band 3.

Syk and Zap-70 Also Exhibit Distinct Enzymatic Activities in Hemopoietic Cells—To verify that these differences also existed for endogenous Syk and Zap-70 molecules expressed in hemopoietic cells, similar experiments were conducted using hemopoietic cell lines. Preliminary studies had shown that the mouse B-cell lines WEHI-231 and A20/2J expressed Syk, but not Zap-70, while the mouse T-cell line BI-141 contained Zap-70, but not Syk (data not shown; Fig. 2A). Unfortunately, we were not able to identify any mouse cell line that exclusively contained SykB. Following cell lysis, Syk and Zap-70 were immunoprecipitated using rabbit anti-Syk and anti-Zap-70 sera, respectively. As controls, Syk, Zap-70, and their Myc-tagged versions were also immunoprecipitated from transiently transfected COS-1 cells using the same antisera. Immune complex kinase reactions (Fig. 6, top panel) were performed as described earlier, and the abundance of Syk and Zap-70 was monitored by immunoblotting of parallel immunoprecipitates with anti-Syk or anti-Zap-70 antibodies (middle panel) or anti-Myc antibodies (bottom panel). These studies showed that Syk from WEHI-231 (lane 1) or A20/2J (lane 2) B-cells had a kinase activity comparable with that of Syk (lane

[Fig. 4. Enzymatic activity of Syk family kinases in immune complex kinase reactions. Myc-tagged proteins were recovered by immunoprecipitation from equivalent amounts (100 μg) of lysates of transiently transfected COS-1 cells, using mAb 9E10. Enzymatic activity was assayed by immune complex kinase reactions, in the presence of 1 μg of the cytosolic domain of erythrocyte band 3 (cdb3; top panel). Reactions were conducted for the indicated periods of time, as outlined under “Materials and Methods.” The abundance of the various Syk family kinases was monitored by anti-Myc immunoblotting of parallel immunoprecipitates, subjected to kinase reactions in the absence of radioactive ATP (bottom panel). The position of the Syk family kinases is indicated by an arrow on the left. The location of cdb3 is also shown on the left, whereas those of prestained molecular mass markers are indicated on the right. Exposures: top panel, 5 min; bottom panel, 14 h.]

[Fig. 5. Effect of depletion of tyrosine-phosphorylated proteins on the activity of Syk family kinases in vitro. A, anti-phosphotyrosine immunoblot. Phosphotyrosine-containing proteins were removed from lysates of transiently transfected COS-1 cells by immunoprecipitation with anti-phosphotyrosine antibodies. The extent of tyrosine phosphorylation of the residual Syk family kinase molecules was determined by anti-phosphotyrosine immunoblotting of anti-Myc immunoprecipitates (top panel). The abundance of Myc-tagged polypeptides was ascertained by anti-Myc immunoblotting of parallel immunoprecipitates with anti-Myc antibodies (bottom panel). The migrations of Myc-Syk and Myc-SykB are shown on the left, while those of prestained molecular mass markers are indicated on the right. Exposures: top panel, 15 h; bottom panel, 2 h. B, immune complex kinase reactions. As in Fig. 5A, except that immunoprecipitates were subjected to immune complex kinase reactions, in the presence of the cytoplasmic domain of band 3 (cdb3). Reactions were conducted for 5 min. The position of the Syk family kinases is indicated by an arrow on the left. The location of cdb3 is also shown on the left, whereas those of prestained molecular mass markers are indicated on the right. Exposure, 5 min.]
The Differential Enzymatic Activity of Syk and Zap-70 Is Determined by Their Catalytic Domain—Several structural domains have been identified in Syk family kinases: two amino-terminal SH2 sequences, a linker region, a catalytic domain, and a carboxyl-terminal tail (Fig. 1A; reviewed in Ref. 1). Whereas the sequence of the SH2 regions and the kinase domain of these enzymes is highly conserved, that of their linker region and carboxyl-terminal tail is more divergent. To understand the structural basis for the biochemical differences noted above, chimeras between Syk and Zap-70 were created by polymerase chain reaction. In the first set of chimeras, the kinase domain and carboxyl-terminal tail of Syk were exchanged for those of Zap-70 and vice versa (Fig. 7). In all cases, the chimeric proteins also possessed the Syk epitope at their amino terminus. The resulting polypeptides were termed Myc-Syk/Zap and Myc-Zap/Syk.

Like Myc-Syk (Fig. 8A, lane 1) and Myc-SykB (lane 2), the Myc-Zap/Syk chimera (lane 5) was prominently tyrosine-phosphorylated in COS-1 cells. On the contrary, Myc-Syk/Zap (lane 4) contained no appreciable amount of phosphotyrosine. Similarly, Myc-Zap/Syk (Fig. 8B, lane 6) provoked an increase in tyrosine phosphorylation of c-Hbl, while Myc-Syk/Zap (lane 5) did not. Finally, in immune complex kinase assays (Fig. 8C), the activity of Myc-Zap/Syk (lane 5) was found to be comparable with that of Myc-Syk (lane 1) and Myc-SykB (lane 2). In contrast, Myc-Syk/Zap (lane 4) had a significantly lower activity, which was even lower than that of Myc-Zap-70 (lane 3).

To identify which element(s) in the carboxyl-terminal half of Syk was (were) required for its elevated kinase activity, a second series of chimeras was generated. In these chimeric proteins, the carboxyl-terminal tails of Syk and Zap-70 were exchanged, thereby creating Myc-Syk/Zap-tail and Myc-Zap/Syk-tail (Fig. 7). Immune complex kinase reactions revealed that transfer of the carboxyl terminus of Syk onto Zap-70 was insufficient to elevate the catalytic activity of Zap-70 (Fig. 9A, top panel, lane 5). Intriguingly, replacement of the tail of Syk by that of Zap-70 reduced its activity in vitro by $2-3$-fold (lanes 4 and 6). However, Myc-Syk/Zap-tail (Fig. 9B, top panel, lane 4) was at least as efficient as Myc-Syk (lane 2) at inducing tyrosine phosphorylation of p120$^{c-$ Syk. While the basis for this discrepancy is not known, it is possible that Myc-Syk/Zap-tail was more susceptible than Myc-Syk to denaturation during cell lysis, thereby explaining its lower activity in in vitro assays. Alternatively, replacement of the carboxyl-terminal tail of Syk by that of Zap-70 may modify the substrate specificity of Syk, rendering it more efficient at phosphorylating c-Hbl, while it was less apt at phosphorylating itself or band 3. In any case, these experiments clearly established that the catalytic domain, and not the carboxyl-terminal tail of Syk, was responsible for its superior activity in COS-1 cells.

**DISCUSSION**

In this manuscript, we have compared the enzymatic activity of Syk, SykB, and Zap-70. To facilitate our analyses, a Myc epitope was added to the amino terminus of all three molecules, allowing their recognition by the same antibody (mAb 9E10). In transiently transfected COS-1 cells, Myc-Syk and Myc-SykB provoked tyrosine phosphorylation of cellular proteins, including Myc-Syk and Myc-SykB themselves. In contrast, Myc-Zap-70 had no effect. In a similar way, Myc-Syk and Myc-SykB, but not Myc-Zap-70, promoted tyrosine phosphorylation of p120$^{c-$ Syk, a known tyrosine phosphorylation substrate expressed in hemopoietic cells (37, 38). Finally, we found that Myc-Syk and Myc-SykB had a $100$-fold greater ability to autophosphorylate and phosphorylate the exogenous substrate erythocyte band 3 in vitro, when compared with Myc-Zap-70. Identical results were obtained when a human zap-70 cDNA was transiently expressed in COS-1 cells.²

To verify that these differences were not restricted to COS-1 cells, the enzymatic activity of Syk and Zap-70 was also assayed using molecules immunoprecipitated from hemopoietic cells. The results of our analyses showed that Syk from COS-1 cells and from different B-cell lines (WEHI-231 and A20/2J) had a $100$-fold greater ability than Zap-70 to autophosphorylate and phosphorylate the exogenous substrate erythocyte band 3 in vitro. Moreover, some molecules were expressed at much higher levels than others, suggesting that Syk is a more efficient tyrosine kinase than Zap-70.

² S. Latour, L. M. L. Chow, and A. Veillette, unpublished data.
2J) had comparable PTK activity in vitro. Furthermore, the activity of Zap-70 from COS-1 cells was similar to that of Zap-70 molecules isolated from a T-cell line (BI-141). Hence, the kinase activity associated with Syk molecules obtained from different sources was greater than that of Zap-70.

What is the basis for this striking difference? It is worth mentioning that Syk and SykB, but not Zap-70, were also tyrosine-phosphorylated in COS-1 cells. Nevertheless, it is im-

**FIG. 8.** Enzymatic function of Syk/Zap and Zap/Syk. The enzymatic function of Myc-Syk/Zap and Myc-Zap/Syk was compared with that of Myc-Syk, Myc-SykB, and Myc-Zap-70. A, tyrosine phosphorylation of Myc-tagged proteins. The extent of tyrosine phosphorylation of the chimeras was determined by immunoblotting of anti-Myc immunoprecipitates with anti-phosphotyrosine antibodies (top panel), as outlined in the legend of Fig. 3A. The abundance of the polypeptides was ascertained by anti-Myc immunoblotting of parallel immunoprecipitates (bottom panel). The migration of the various Myc-tagged polypeptides is highlighted by an arrow on the left. The position of heavy chain of Ig is shown on the left, while those of prestained molecular weight markers are indicated on the right. Exposures: top panel, 2 h; bottom panel, 2 h. B, tyrosine phosphorylation of c-Cbl. COS-1 cells were transfected with the indicated DNAs, in the presence of a mouse c-cbl cDNA. c-Cbl was recovered by immunoprecipitation from 250 μg of cellular proteins and probed by immunoblotting with anti-phosphotyrosine antibodies (top panel). The abundance of c-Cbl was determined by stripping the immunoblot and re-hybridizing with anti-Cbl antibodies (bottom panel). The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular mass markers are shown on the right. Exposures: top panel, 6 h; bottom panel, 4 h. C, immune complex kinase assays. Myc-tagged proteins were immunoprecipitated with mAb 9E10 from 100 μg of total cellular proteins. Kinase reactions were conducted for 5 min, in the presence of the cytoplasmic domain of erythrocyte band 3 (cdb3). The position of the Syk family kinases is indicated by an arrow on the left. The location of the cytoplasmic domain of erythrocyte band 3 (cdb3) is also shown on the left, whereas those of prestained molecular mass markers are indicated on the right. Exposure, 5 min.

**FIG. 9.** Enzymatic function of Syk/Zap-tail and Zap/Syk-tail. As in the legend of Fig. 8, except that Myc-Syk/Zap-tail and Myc-Zap/Syk-tail were evaluated. A, immune complex kinase reactions. Myc-tagged proteins were immunoprecipitated from 10 or 20 μg of total cellular proteins, as indicated. Kinase reactions were conducted for 2 min, in the presence of the cytoplasmic domain of erythrocyte band 3 (cdb3). The position of the Syk family kinases is indicated by an arrow on the left. The location of the cytoplasmic domain of erythrocyte band 3 (cdb3) is also shown on the left, whereas those of prestained molecular mass markers are indicated on the right. Exposures: top panel, 7 min; bottom panel, 1.5 h. B, tyrosine phosphorylation of c-Cbl. The migrations of c-Cbl and heavy chain of Ig are indicated on the left; those of prestained molecular mass markers are shown on the right. Exposures: top panel, 2 h; middle panel, 1.5 h; bottom panel, 6 h.
probable that this phosphorylation explained the superior activity of Syk and SykB, as depletions of tyrosine-phosphorylated polypeptides did not alter the relative activity of these molecules in kinase reactions. In other experiments, we also addressed the possibility that a cellular PTK bound to Syk, but not to Zap-70, explained the greater kinase activity found in anti-Syk immunoprecipitates. However, our results provided firm evidence that this activity was intrinsic to Syk, as it was abolished by mutation of the Syk ATP-binding site (data not shown). Finally, the structural basis for this difference in activity of Syk and Zap-70 was determined by constructing chimeras between these two kinases. Our analyses showed that the catalytic sequences of these enzymes, but not their SH2 domains, linker region or carboxyl-terminal tail, conferred their distinct capacities to phosphorylate substrates in vitro and in vivo. While it is likely that Syk has a greater overall PTK activity than Zap-70, it could not be completely ruled out that Syk and Zap-70 actually have distinct substrate specificities. Perhaps Zap-70 is active only toward a limited set of cellular substrates. This possibility is unlikely, however, as Syk was consistently more active than Zap-70 in vitro, under a variety of assay conditions (data not shown). Furthermore, Syk was more efficient than Zap-70 at phosphorylating a variety of PTK substrates in COS-1 cells, including c-Cbl (this report), phospholipase C-ε1, Slp-76, Shc, and HS1.3

These findings may provide an explanation for the dissimilar impacts of Syk and Zap-70 on cell signaling, which were noted in earlier studies (20, 39). Antibody-mediated aggregation of a CD16-CD7-Syk chimera, but not of a CD16-CD7-Zap-70 chimera, was sufficient to induce cytolytic functions in a human CD16-CD7-Syk chimera, but not of a CD16-CD7-Zap-70 chimera, was sufficient to induce cytolytic functions in a human T-cell line (20). Similarly, the Syk chimera was more efficient than the Zap-70 chimera at triggering phagocytosis in COS-1 cells (40). It is plausible that Syk performed better than Zap-70 in these systems, because it has a superior intrinsic PTK activity. Since co-aggregation of the Zap-70 chimera with a Fyn or Lck chimera was needed for activation of T-cell cytotoxic functions (20), it has been postulated that the function of Zap-70, but not that of Syk, absolutely requires activation of Src family kinases. This concept is in keeping with the observation that phosphorylation of Zap-70 at tyrosine 493 by Src family kinases increased its catalytic activity in vitro (41, 42) and was necessary for Zap-70-mediated functions in vivo (41). In spite of these differences, Gong et al. (43) observed that Syk and Zap-70 were equally efficient at rescuing antigen receptor-induced signals in Syk-deficient DT-40 chicken B-cells. While this finding may seem to contradict our data, we feel that it indicated that, in the context of the other post-translational modifications triggered by antigen receptor stimulation, Zap-70 can become able to transduce signals as efficiently as Syk. Possibly, the biological activity of Zap-70 is maximized as a consequence of its binding to tyrosine-phosphorylated ITAMs and/or its tyrosine phosphorylation by activated Src family kinases.

Collectively, these observations suggest that the prerequisites for signal transduction by Syk and Zap-70 differ strikingly. While both associate with tyrosine-phosphorylated ITAMs, the higher intrinsic PTK activity of Syk may allow this enzyme to induce intracellular protein tyrosine phosphorylation more readily. Perhaps, Syk-mediated signals can even occur in the absence of activation of Src family members and binding to ITAMs. In contrast, Zap-70-mediated signal transduction may be fully dependent on adequate activation of Src-related PTKs and association with tyrosine-phosphorylated ITAMs. Such a distinction would presumably permit a tighter control of Zap-70-mediated signals, which may be crucial to avoid inappropriate T-cell antigen receptor signaling. Even though the biological significance of these differences remains to be firmly established, it is provocative that T-cells can express both Syk and Zap-70, in a developmentally regulated manner (5). The relative abundance of Syk may dictate the requirement for activated Src family kinases to initiate antigen receptor signaling and be a crucial determinant of T-cell responsiveness.

SykB is an alternative Syk isoform previously identified by cDNA cloning in the human T-cell leukemia line Jurkat (35) and the rat basophilic leukemia line RBL-2H3 (Ref. 36). While the SykB protein was shown to accumulate in RBL-2H3 cells (36), its expression was not documented in normal cells in this earlier study. Herein, we report the isolation of a sykB cDNA from mouse thymus. Ribonuclease protection assays showed that ~10% of sykB RNAs in thymus, spleen, and a variety of hemopoietic cell lines was of the sykB configuration. However, we were unable to unabashedly document expression of the SykB protein in these cells. But we found that sykB represented ~50% of all sykB transcripts and polypeptides present in normal mouse bone marrow cells. Even though the exact cell type(s) containing sykB remain(s) to be determined, our data nonetheless established that SykB is a genuine Syk isoform that may have a unique role in bone marrow cell signaling. Whereas our biochemical analyses indicated that Syk and SykB exhibit comparable intrinsic PTK activity, it is plausible that the deletion in the linker region removes binding sites for other cellular proteins (2), or modifies the conformation of Syk, thereby changing its function and/or regulation. Obviously, future studies are indicated to determine the role of this Syk isoform in hemopoietic cells.

In summary, we have shown that the intrinsic PTK activity of Syk and SykB is significantly greater than that of Zap-70 in transiently transfected COS-1 cells and in hemopoietic cells. Through structure-function analyses, it was also established that this biochemical difference relates to sequence variations in the catalytic domain of the two enzymes. While the full physiological significance of these observations remains to be established, they imply that Syk is more readily efficient at transducing signals in hemopoietic cells. In contrast, the participation of Zap-70 may require additional post-translational modifications, such as binding to tyrosine-phosphorylated ITAMs and/or phosphorylation by Src family kinases. These hypotheses are currently being tested experimentally.

Acknowledgments—We thank A. Shaw for his kind gift of the zap-70 cDNA and the vector containing the Mye epitope, P. Low for provision of the expression vector for erythrocyte band 3, Ron Wong and Larry Samelson for purified band 3 and useful advice of its purification, and Lou Matis for gift of the fetal thymus cDNA library. We also acknowledge D. Davidson for her critical reading of the manuscript.
Enzymatic Activities of Syk and Zap-70

Differential Intrinsic Enzymatic Activity of Syk and Zap-70 Protein-tyrosine Kinases
Sylvain Latour, Lionel M. L. Chow and André Veillette

doi: 10.1074/jbc.271.37.22782

Access the most updated version of this article at http://www.jbc.org/content/271/37/22782

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 42 references, 27 of which can be accessed free at http://www.jbc.org/content/271/37/22782.full.html#ref-list-1