Immature myeloid cells have been shown to transduce signals through a carboxy-terminally truncated isoform of Stat5. This functionally distinct signal transducer and activator of transcription isoform is generated through a unique protein-processing event. Evaluation of numerous cell lines has determined that there is a direct correlation between the expression of truncated Stat5a and protease activity. Moreover, protease activity is found only in the myeloid and not in lymphoid progenitors. To further characterize the protease small quantities have been purified to near homogeneity. Studies on this purified material indicate that the protease has an apparent molecular mass of ~25 kDa and is active over a wide range of pH values. The protease will also cleave both activated (i.e. tyrosine-phosphorylated) and inactivate Stat5. Although this activity is sensitive to phenylmethylsulfon fluoride, it is notably not sensitive to several other serine protease inhibitors. Additional studies have led to the identification of the unique site where the protease cleaves Stat5. Mutagenesis of this site renders Stat5 resistant to cleavage. Consistent with the model that Stat5 cleavage is important for early myeloid development, introduction of a “non-cleavable” isoform of Stat5 into FDC-P1 cells (a myeloid progenitor line) leads to significant phenotypic changes.

Characterization of the ability of IFNs to induce genes rapidly has led to the identification of the JAK-STAT pathway (1–3). In this signaling paradigm, JAKs are receptor-associated tyrosine kinases, and STATs (signal transducers and activators of transcription) are the cytoplasmic transcription factors they activate. Once activated, STATs dimerize, translocate to the nucleus, and bind to enhancer elements, culminating in gene induction. Subsequent studies have determined that all members of the cytokine family transduce signals through one or more of the seven members of the STAT family (4, 5). These STATs share several functionally conserved domains including an amino-terminal coiled-coil domain, a DNA binding domain, a linker domain, an SH2 domain, a tyrosine activation domain, and a divergent carboxy-terminal transcriptional activation domain (6, 7).

Interleukin (IL)-3 is a member of a subfamily of functionally related cytokines (i.e. IL-3, IL-5, and GM-CSF) that all signal through a common receptor chain (8, 9). Consistent with this, all three ligands play an important role in the maturation, proliferation, and activation of myeloid lineages (10). Moreover, a number of studies have determined that these ligands transduce signals through several isoforms of Stat5. In most cell types, full-length Stat5a (96 kDa) and Stat5b (94 kDa) are activated in response to stimulation with IL-3 or other members of this family (1–3). This leads to the induction of several known Stat5 target genes (11–19). In the absence of both Stat5a and Stat5b, there are significant defects in the development of CFU-Mix, CFU-Eos, and CFU-GM colonies, as well as defects in the induction of target genes (20).

In myeloid progenitors, IL-3 stimulates the induction of carboxy-terminally truncated isoforms of both Stat5a (i.e. 77 kDa) and Stat5b (i.e. 80 kDa). These isoforms, which are missing their transcriptional activation domain, are functionally distinct and fail to promote the induction of Stat5 target genes (19, 21, 22). In contrast to other STATs (23–25), the truncated isoforms of Stat5 are generated through a unique protein-processing event. Previous studies have indicated that this protease is specific for Stat5 and can only be found in several immature cell lines (19).

In this work, we extend these studies and demonstrate that there is a direct correlation between the expression of truncated Stat5 isoforms and protease activity. Moreover, this activity is only found in myeloid progenitors, supporting our hypothesis that the Stat5 protease plays an important regulatory role during myelopoiesis. We find no evidence for this during lymphoid development. Biochemical characterization of this protease has revealed that it has an apparent molecular mass of ~25 kDa and exhibits a unique pattern of sensitivity to serine protease inhibitors. Functional evidence is provided for a role of this protease in myeloid development.

MATERIALS AND METHODS

Cell Culture—Cell culture reagents were purchased from Life Technologies, Inc. DA-3, 32Dc1, WEHI3b, Ba/F3, FdTrk, and HSC15 (a generous gift from J. Pierce) cells were grown as described previously in RPMI supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, and conditioned media as required (1, 24–28). 3T3, 293, and HeLa cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented either with 10% fetal calf serum (3T3 and 293 cells) or calf serum (HeLa cells). Thymocytes were prepared by mechanical disruption of harvested thymuses as previously reported (28). Prior to treatment with cytokines, cells were starved of growth factors and/or serum for 4–6 h. DA-3, 32Dc1, WEHI3b, Ba/F3, cl.19 (an FDC-P1 derivative (29, 30), and FdTrk (an FDC-P1 derivative (1) cells were either stimulated with 10% WEHI3b conditioned media or IL-3 (10 units/ml; Peprotech). FDC-P1 (cl.19) cells, a generous gift of L. Rohrschneider, were differentiated by stimulation with murine GM-CSF (10 units/ml; Peprotech) for 3 days, as previously reported (29, 30).
HSC15 cells and thyromes were stimulated with IL-7 (10 units/ml; Genzyme).

Plasmids—The R10 chimeric receptor represents a modest variation of Gαsα, where the ecto- and transmembrane domains of the human G-CSF receptor are fused to a truncated IFN-γ membrane domain from the human IFN-γ receptor 1 chain (IFNAR1). In R10, the ITAM motif has been substituted with the Stat5 recruitment cassette from the human IL-2 receptor β-chain (NDTAYLSQELQ (32)). Stat5b protein expression studies were generated through site-directed mutagenesis (Quick-Change; Stratagene) of a Stat5b RCMV (Invitrogen)-driven expression construct (19), with the following pairs of oligonucleotides (Operon): S5CSm4u: TCCACAgATgCTgggAgTggCgCCACCgCCATggATCAggCTCCTTCC; S5CSmtu, ggAaggAggCTtgATCtgCggCggCCACCTAggCATTgT; S5CSm2u, gAgtgAggCTtgCggCggCCACCTAggCATTgT; S5CSm1l, ggAggAggCCtccAggCAGgtCggCCACCTAggCATTgT; and S5CSm1i, gAgtgAggCTtgCggCggCCACCTAggCATTgT. The Stat5bm/m mutant (i.e. Y724S; M725A) was also cloned into MSCV (a generous gift of D. Dusart-Four) (35) for stable expression in FDC-P1 cells.

Protease Assays—Stat5 cleaving activity was evaluated either through immunoblotting or electrophoretic mobility shift assay (EMSA). Briefly, 1 μl of recombinant Stat5b (or Stat5a) substrate (prepared by transient transfection; see above) was incubated with 1-5 μl of crude or partially purified protease for 30–60 min at 37 °C. The reagents were then evaluated either by SDS-PAGE or EMSA with an IFR1-GAS probe (gatcATTGCCCCGAAAT; Oligo Inc.) described previously (1, 38). For pH optima studies, either 1 μl of Heps (pH 7.4), 2 μl of EDTA, 1 μl of dithiothreitol, or 1 μl of dithiothreitol, 10% glycerol (19, 35) or Nonident-P-40 buffer (0.5% Nonident-P-40, 20 μm Tris (pH 7.5), 150 mM NaCl, 10% glycerol (27)) as indicated. For PDC-F1 (cl.19) cells were transfected by electroporation (Gentronics model ECM600; 260 V, 1050 farads with infinite resistance), indicated in the figure legend, were pooled, diluted to 150 mM NaCl, and applied to a 50-ml size exclusion column (2 × 27 cm; SE100/40) and a second 3-ml DEAE-MacroPrep column (Bio-Rad), equilibrated to pH 8.4. A concentrated sample of Stat5a and Stat5b (i.e. p77 and Stat5b (i.e. 80), was found in nuclear extracts prepared from immature cell types (1, 22). A slower migrating complex, consisting of the full-length isoforms of Stat5a (i.e. p96) and Stat5b (i.e. p94), is found in most other cells (1, 2). The fast migrating complex has been well characterized in DA-3 and FDC-P1 cells, which represent myeloid progenitors (1, 19, 39–41). This complex has also been reported in other early myeloid cell lines, including 32Dc1 (27). But a more differentiated subline of 32Dc1 cells, which exhibits less potent protease activity (i.e. see Fig. 1, lane 2), yields only the full-length complex after stimulation with IL-3 or erythropoietin (27).

Recent studies have determined that a protease is responsible for the generation of the truncated isoforms of Stat5 in DA-3 and FDC-P1 cells (19, 41). To extend these initial observations, extracts were prepared from several additional cell lines and evaluated for Stat5 protease activity (Fig. 1). In the absence of protease activity, the full-length DNA binding activity (i.e. an "Intact" p94 homodimer) was recovered. If protease activity was present, then the full-length recombinantactivated Stat5b (i.e. rStat5b) was cleaved into the faster migrating "truncated" DNA binding activity (i.e. the "Cleaved" p80 homodimer). Extracts prepared from FdTrk cells served as the positive control for protease activity (19) (see Fig. 1, lane 2).

In several cases, an additional intermediate complex (i.e. a p94:p80 heterodimer; data not shown), which correlated with less potent protease activity (e.g. see Fig. 3A), was recovered (see Fig. 3A, lanes 3–5). Similar results were obtained with the slower migrating (19), recombinant, activated Stat5a (rStat5a; see Fig. 1, lanes 10 and 11).

Stat5 protease activity was recovered from each of four cell lines where truncated Stat5 had previously been identified (i.e. FdTrk, WEHI-3b, DA-3, and 32Dc1 (1, 19, 27)). Yet, in four cell lines, where only full-length Stat5 had previously been identified (i.e. 293, Baf3/ HelA, and ST3 cells (1, 19, 27, 43)), rStat5b was still detectable (see Fig. 1, lanes 6–9). As described previously (1, 19, 22, 27), the truncated species of Stat5a to Stat5b and the more robust levels of DNA binding activity than the full-length isoform. An important control for these studies, evaluation of the intrinsic DNA binding activity recovered from each of the CHAPS extracts (Fig. 1, lanes 12–19), demonstrated that there was an insufficient level of endogenous Stat5 DNA binding activity to confound the results. These studies demonstrate...
that there is a direct correlation between the activation of the carboxyl-terminally truncated isoforms of Stat5 and the presence of Stat5 protease activity.

Signaling in Lymphocytes—Analogous to myelocytes, the development of lymphocytes is a multistep process that is carefully regulated. Two cytokines that are critical to lymphoid development, IL-2 and IL-7, transduce their signals through Stat5 (20, 32, 44–48). To determine whether the truncated species of Stat5 may also be differentially activated in developing lymphocytes, extracts were prepared from cell lines representing several stages in lymphoid development (e.g. 22D6, 1881, S49, AKR and P3X (49–52)). In each case, stimulation with IL-7 led to the formation of the full-length DNA binding complexes (data not shown), indicating that the activation of a carboxyl-terminally truncated Stat5 isoforms is not important for these cells. Reasoning that the truncated isoform of Stat5 may be activated at an earlier developmental stage than those represented by these cell lines, lymphocytes from RAG-1 null mice were evaluated. As RAG-1 is important in immunoglobulin and T-cell receptor chain rearrangements, these mice exhibit an earlier block in T-cell and B-cell maturation (53).

To evaluate Stat5 activation in these early lymphocytes, thymic T-cells were collected from both RAG-1 knock-out mice (predominantly CD44+/CD25- /CD4+ /CD8-) and control C57Bl/6J mice (predominantly CD44+/CD25-/CD4+/CD8+) and then stimulated with IL-7. DNA binding activity was evaluated by EMSA (see Fig. 2). Stat5 DNA binding activity for IL-2-stimulated BaF/3 cells and FdTrk cells served as controls for the slower (i.e. intact) and faster (i.e. cleaved) migrating forms of Stat5. In each case, IL-7 induced a Stat5 DNA binding complex that comigrated with the triplet of bands representing full-length Stat5 (i.e. p94:p94, p94:p96, and p96:p96 dimers (1, 2)). Similar results were obtained when these extracts were evaluated by immunoblotting with a pan-Stat5 reactive antibody (data not shown). Therefore, in contrast to studies in myelocytes, there is no evidence that truncated isoforms of Stat5 play an important role in transducing signals during the examined stages of lymphoid development.

Fig. 1. Immature myeloid cell lines express Stat5 protease activity. Recombinant activated (*) Stat5b (rSt5b*) or Stat5a (rSt5a*), prepared from 293 cells, was incubated with CHAPS extracts prepared from each of the indicated cells lines (lanes 2–11) at 37 °C for 1 h and then evaluated by EMSA with an IRF-1 GAS probe. The same extracts were also evaluated by EMSA without incubation with recombinant Stat5 (Extracts Alone, lanes 12–19). The mobilities of the DNA binding complex representing Intact (i.e. p94:p94) and Cleaved (i.e. p80:p80) are indicated in the left margin. p80-p94 represents an intermediate complex.

Fig. 2. Stat5 activation on lymphocytes. Whole cell extracts prepared from thymocytes harvested from 6- to 7-week-old RAG-1+/− mice (lanes 3 and 4) or control C57Bl/6J mice (lanes 5 and 6) either before (lanes 3 and 5) or after (lanes 4 and 6) stimulation with IL-7 were evaluated by EMSA as outlined for Fig. 1. IL-3-stimulated BaF/3 cells serve as a positive control for full-length Stat5 isoforms (Intact, lane 1) and IL-3-stimulated FdTrk cells as a positive control for truncated Stat5 isoforms (Cleaved, lane 2). IL-7-stimulated HSIC15 cells (a pre-B-cell line) serve as a positive control for IL-7 stimulation (lanes 7 and 8).
protease. In the most active fraction from the size exclusion column (i.e. fraction 31), 1–2 faint bands of ~25 kDa not found in inactive fractions (e.g. fraction 24) could be visualized, especially after concentration. Consistent with this, a single major ~25-kDa band was identified when the most active fraction (A) from the second DEAE-chromatography step was concentrated. This band was not evident in inactive fractions (I). These data indicate that the protease is likely to be a ~25-kDa protein.

The Stat5 Protease Is Active in a Wide Range of Physiological pH Values—Optimizing several of the chromatographic steps required understanding whether the Stat5 protease was sensitive to fluctuations in pH. To determine this, the pH of protease reactions was adjusted with a 30-fold molar excess of Hepes or Tris buffer. To increase the sensitivity of this assay, smaller volumes of protease and shorter incubations were employed. As shown in Fig. 4, the protease was able to cleave Stat5 under pH values ranging from 6.0 to 8.6. Although the protease has potent activity at each of these pH values, cleaving activity was modestly diminished in the pH 6.0 (Hepes) or pH 6.7 (Tris) samples. These studies suggest that the Stat5 protease is active in a wide range of physiological pH values.

The Stat5 Protease Is a Member of the Serine Family of Proteases—Previous studies, which had determined that the Stat5 protease was sensitive to PMSF, suggested it was a member of the serine family of proteases. To evaluate this more carefully, digestions were carried out in the presence of several additional protease inhibitors. Of these, only PMSF (170 \( \mu \)g/ml) significantly inhibited Stat5 protease activity (see Fig. 5). PMSF is a serine protease inhibitor with broad specificity. It also has some activity against several cysteine proteases (e.g. papain). In contrast to the potent activity of PMSF, leupeptin (0.5 \( \mu \)g/ml), another serine/cysteine protease inhibitor (also active against papain), had no effect on Stat5 cleaving activity. Likewise, apritinin (2 \( \mu \)g/ml), a serine-specific protease inhibitor, and pepstatin (0.7 \( \mu \)g/ml), an aspartic protease inhibitor, did not block Stat5 protease activity. However, partial inhibition was obtained with DFP, another potent serine protease inhibitor (data not shown (54)). Several additional protease inhibitors, including benzamidine (a serine-specific inhibitor), E-64 (a cysteine-specific inhibitor), and EDTA (a metalloprotease inhibitor), were also ineffective at blocking protease activity (data not shown). This pattern of inhibition indicates that
the Stat5 protease is an unusual member of the serine family of proteases.

The Stat5 Protease Cleaves Activated and Inactivated Isoforms of Stat5 Equivalently—The cleavage assays employed to date only examined the ability of the Stat5 protease to cleave activated (i.e. tyrosine-phosphorylated) Stat5. However, as this protease can be isolated from unstimulated cells (see Fig. 1), it was of interest to determine whether it could cleave nonphosphorylated Stat5 as well. To test this, recombinant Stat5b was prepared both before and after stimulation of a cotransfected receptor (31). As anticipated, activated Stat5b bound DNA and was detected by an antiphosphotyrosine-specific antibody. In contrast, Stat5b overexpressed in unstimulated cells failed to bind DNA (see below (19)) and was not recognized by an antiphosphotyrosine-specific antibody (data not shown). When cleavage was evaluated by an immunoblotting assay, it was evident that both native and tyrosine-phosphorylated Stat5b were cleaved equally well by partially purified preparations of protease (Fig. 6). These observations indicate that the protease may both cleave Stat5 constitutively (i.e. in unstimulated cells) and after activation (i.e. in stimulated cells).

The Stat5 Protease Cleavage Site—Through both sequence analysis of purified p77 and p80 peptides (1), and subsequent mapping studies (19), the cleavage site in Stat5 has been mapped distal to amino acid 720 (i.e. in Stat5b). To map more carefully the cleavage site, biotinylated peptides spanning this region of Stat5a and Stat5b (i.e. amino acids 719–730 and 724–735, respectively) were generated (see Fig. 7A). The sequence of the carboxyl-terminal peptide of Stat5b was determined, before or after cleavage with partially purified preparations of protease (1 h at 37 °C), as determined by MALDI mass spectroscopy. The conserved residues in the cleavage site are boxed. The sequence of the carboxyl-terminal peptide of Stat5b obtained from purified Stat5b (1) is underlined. B, the five mutations introduced into the full-length Stat5b cDNA, including m/m, m1, m2, m3, and m4, as well as the corresponding wild type (wt) sequence, are indicated.

Fig. 6. Activated and inactivated Stat5 are both cleaved by the Stat5 protease. Stat5b was prepared from 293 cells either before (−) or after stimulation (+) of the cotransfected chimeric receptor. These preparations of Stat5b were then incubated in buffer or partially purified preparations of protease for 1 h at 37 °C. The digested samples were fractionated on an 8% SDS-PAGE and evaluated by immunoblotting with a pan-Stat5 antibody (Signal Transduction Laboratories).

A

|      | Control | Control | Protease | Protease | Protease Alone |
|------|---------|---------|----------|----------|----------------|
| lane 1 |          |          |          |          |                |
| lane 2 |          |          |          |          |                |
| lane 3 |          |          |          |          |                |
| lane 4 |          |          |          |          |                |
| lane 5 |          |          |          |          |                |

B

|       | Mu5b/1  | Mu5b/m  | Mu5b/m  | Mu5b/m  | Mu5b/m  |
|-------|---------|---------|---------|---------|---------|
| lane 1 |         |         |         |         |         |
| lane 2 |         |         |         |         |         |
| lane 3 |         |         |         |         |         |
| lane 4 |         |         |         |         |         |
| lane 5 |         |         |         |         |         |

The cleavage site for the Stat5-specific protease. A, homologous regions of amino acid sequence from the human (Hu) and murine (Mu) Stat5a and Stat5b gene products and the protease cleavage site are indicated in the top half of this panel. A synthetic biotinylated Stat5a peptide (spanning amino acids 714–725) and Stat5b peptide (spanning amino acids 719–730) are indicated in the bottom half of this panel (synthesized at Amgen, Boulder, CO). Included are the masses and sequences of each of the peptides before or after cleavage with partially purified preparations of protease (1 h at 37 °C), as determined by MALDI mass spectroscopy. The conserved residues in the cleavage site are boxed. The sequence of the carboxyl-terminal peptide of Stat5b obtained from purified Stat5b (1) is underlined. B, the five mutations introduced into the full-length Stat5b cDNA, including m/m, m1, m2, m3, and m4, as well as the corresponding wild type (wt) sequence, are indicated.

Fig. 7. Cleavage site for the Stat5-specific protease. A, homologous regions of amino acid sequence from the human (Hu) and murine (Mu) Stat5a and Stat5b gene products and the protease cleavage site are indicated in the top half of this panel. A synthetic biotinylated Stat5a peptide (spanning amino acids 714–725) and Stat5b peptide (spanning amino acids 719–730) are indicated in the bottom half of this panel (synthesized at Amgen, Boulder, CO). Included are the masses and sequences of each of the peptides before or after cleavage with partially purified preparations of protease (1 h at 37 °C), as determined by MALDI mass spectroscopy. The conserved residues in the cleavage site are boxed. The sequence of the carboxyl-terminal peptide of Stat5b obtained from purified Stat5b (1) is underlined. B, the five mutations introduced into the full-length Stat5b cDNA, including m/m, m1, m2, m3, and m4, as well as the corresponding wild type (wt) sequence, are indicated.

Fig. 5. Stat5 protease sensitivity to protease inhibitors. Recombinant activated Stat5b, prepared from 293 cells, was incubated with partially purified preparations of protease for 1 h at 37 °C in the absence (lane 1) or presence of protease inhibitors pepstatin (Sigma; 0.7 µg/ml; lane 2), aprotinin (Sigma; 2.0 µg/ml; lane 3), PMSF (Roche Molecular Biochemicals; 170 µg/ml; lane 4), and leupeptin (Sigma; 0.5 µg/ml; lane 5). Samples were evaluated by EMSA with an IRF-1 GAS probe.

Fig. 7. Cleavage site for the Stat5-specific protease. A, homologous regions of amino acid sequence from the human (Hu) and murine (Mu) Stat5a and Stat5b gene products and the protease cleavage site are indicated in the top half of this panel. A synthetic biotinylated Stat5a peptide (spanning amino acids 714–725) and Stat5b peptide (spanning amino acids 719–730) are indicated in the bottom half of this panel (synthesized at Amgen, Boulder, CO). Included are the masses and sequences of each of the peptides before or after cleavage with partially purified preparations of protease (1 h at 37 °C), as determined by MALDI mass spectroscopy. The conserved residues in the cleavage site are boxed. The sequence of the carboxyl-terminal peptide of Stat5b obtained from purified Stat5b (1) is underlined. B, the five mutations introduced into the full-length Stat5b cDNA, including m/m, m1, m2, m3, and m4, as well as the corresponding wild type (wt) sequence, are indicated.
However, in contrast to wild type Stat5b (Stat5b wt), it was highly resistant to cleavage. Again, cleaving activity was sensitive to PMSF. Similar results were obtained when cleavage was evaluated by SDS-PAGE (see Fig. 8B), but this assay also revealed some novel Stat5bm/m cleavage products. These products were more evident in purified protease preparations, suggesting that they may represent overdigestion or perhaps a small loss in specificity. However, as these species are only seen with the Stat5bm/m substrate, they are likely to represent secondary cleavage sites. Once again, this cleaving activity is sensitive to PMSF.

To define the cleavage site more carefully, additional mutants were generated (see Fig. 9B). First, the amino acids flanking the cleavage site, Tyr-724 and Met-725, were individually mutated. Surprisingly, mutant Stat5bm1 (Y724A) exhibited poor DNA binding activity (Fig. 9A, lanes 3 and 4) and could not be evaluated by this assay. In contrast, mutant Stat5bm2 (M725L) exhibited good DNA binding activity (Fig. 9A, lanes 5 and 6) and was readily cleaved but not as well as control Stat5b. The next mutant, Stat5bm3 (T723V), also failed to bind DNA and likewise could not be evaluated by this assay. The final mutant, Stat5bm4 (A722S), exhibited good DNA binding and was readily cleaved. A more complete picture emerged when these cleavage reactions were evaluated by immunoblotting after fractionation on an SDS-PAGE (Fig. 9B). Although Stat5bm1 was less robustly expressed, it was clearly resistant to cleavage (lanes 3 and 4). In contrast, cleavage of each of the other three mutants was comparable to that of rStat5b wt. These studies indicate that Tyr-724 in Stat5b (and by extension Tyr-719 in Stat5a) is critical for cleavage.

Expression of Stat5bm2 (M725L) exhibited good DNA binding activity (Fig. 9A, lanes 5 and 6) and was readily cleaved but not as well as control Stat5b. The next mutant, Stat5bm3 (T723V), also failed to bind DNA and likewise could not be evaluated by this assay. The final mutant, Stat5bm4 (A722S), exhibited good DNA binding and was readily cleaved. A more complete picture emerged when these cleavage reactions were evaluated by immunoblotting after fractionation on an SDS-PAGE (Fig. 9B). Although Stat5bm1 was less robustly expressed, it was clearly resistant to cleavage (lanes 3 and 4). In contrast, cleavage of each of the other three mutants was comparable to that of rStat5b wt. These studies indicate that Tyr-724 in Stat5b (and by extension Tyr-719 in Stat5a) is critical for cleavage.

**Fig. 8. Double mutant Stat5 is resistant to cleavage.** A. extracts prepared from activated 293 cells cotransfected with a chimeric receptor and either wild type Stat5b cDNA (rStat5bwt, lanes 1–3) and doubly mutant Stat5b (rStat5bm/m, lanes 4–6) were incubated with protease (lanes 2, 3, 5, and 6) and evaluated by EMSA as outlined in Fig. 1. This entailed incubation with partially purified preparations of protease for 1 h at 37 °C either in the absence or presence of the inhibitor PMSF. The mobility of p94:p94 homodimer (Intact) and p80:p80 homodimer (Cleaved) complexes are indicated in the margins. B, extracts detailed in A were evaluated by immunoblotting with a panStat5 antibody (Signal Transduction Laboratory) after fractionation on a 7% SDS-PAGE. Molecular weight markers and the mobility of Intact (p94) and Cleaved (p80) Stat5b products are indicated in the left and right margins, respectively.

**Fig. 9. Cleavage of additional Stat5 point mutants.** A, extracts prepared from activated 293 cells cotransfected with a chimeric receptor and either vector (lanes 1 and 2), wild type Stat5b cDNA (rStat5bwt, lanes 11 and 12), or the indicated Stat5b point mutants (rStat5bm1, rStat5bm4, lanes 3–10; see Fig. 7B for details) were assayed by EMSA as outlined in Fig. 1. DNA binding activity was evaluated either before (−) or after (+) a 1-h digestion with partially purified preparations of protease at 37 °C. The mobility of p94:p94 homodimer (Intact) and p80:p80 homodimer (Cleaved) and the intermediate complex, p80:p94, are indicated in the margins. B, extracts detailed in A were evaluated by immunoblotting with a panStat5 antibody (Signal Transduction Laboratory) after fractionation on a 7% SDS-PAGE. Molecular weight markers and the mobility of p94 and p80 are Stat5b products are indicated in the left and right margins, respectively.
Stat5 Protease

**Fig. 10.** FDC-P1 subclones expressing Stat5 mutants. A, phosphotyrosine-Stat5 immunoblot of FDC-P1 sublines expressing either wild type or non-cleavable isoforms of Stat5. Extracts were prepared from cells before (−, i.e., starved) or after (+) stimulation with IL-3, fractionated on 8% SDS-PAGE, and immunoblotted with a Stat5 phosphotyrosine-specific antibody (Upstate Biotechnology Inc.). Lines A1 and A2 represent clones expressing Stat5<sup>bm/m</sup> (lanes 3–9). Line B1 expresses a control Stat5<sup>wt</sup> (lanes 9 and 10). Line D3 represents a neomycin-resistant vector alone control (lanes 3 and 4). Controls for truncated (rStat5b) and full-length (rStat5b) activated Stat5b are provided in [lanes 1 and 2](https://doi.org/10.1074/jem.2019203029). The mobility of p94, p80 (cleaved Stat5b), and p77 (cleaved Stat5a) are indicated in the margins. B, extracts detailed in A were evaluated by immunoblotting with a panStat5 antibody (Signal Transduction Laboratory).

The expression of full-length Stat5 in these cells could be anticipated to promote the expression of more "mature genes," thereby altering the phenotype of these cells. Briefly, neomycin-resistant clones were selected from FDC-P1 (cl.19) cells transfected with either Stat5<sup>b/m</sup>, Stat5<sup>b/wt</sup>, or "vector alone" and then screened by an immunoblotting assay (see Fig. 10). To increase the sensitivity of this assay, nuclear extracts were probed with a Stat5 phosphotyrosine-specific antibody (Fig. 10A). Note that low levels of Stat5, representing cytoplasmic contamination, could be detected in unstimulated extracts (Fig. 10B). But these species were never activated. Four clones were evaluated. Two of the neomycin-resistant clones were recovered from the Stat5<sup>b/m</sup> transfectants (i.e. A1 and A2) and expressed activated full-length Stat5b in nuclear extracts. This contrasted the control vector alone (i.e. D3 [Neo<sup>-</sup>]) and Stat5<sup>b/wt</sup> (B1) transfectant lines, where as anticipated only truncated Stat5 was recovered from the nuclei of stimulated cells (see Fig. 10, [lanes 4 and 10](https://doi.org/10.1074/jem.2019203029)). In the A1 clone (Stat5<sup>b/m</sup>, lane 6), both full-length and truncated Stat5 were present in stimulated nuclear extracts, suggesting that both activated endogenous and mutant Stat5 had translocated to the nucleus. However, in the A2 clone (Stat5<sup>b/m</sup>, lane 8), only full-length Stat5 was identified in stimulated nuclear extracts.

Next, several of these clones were evaluated histochemically. When the D3 clone was cultured in IL-3 (Fig. 11A), it exhibited the characteristic features of immature self-renewing FDC-P1 cells (29, 30). When the D3 clone was cultured in GM-CSF, it acquired characteristic features of differentiation (see Fig. 11B), including increased size, increased (cytoplasmic) granularity, increased nuclear and cytoplasmic volume, and increased membrane ruffling (29, 30). The A1 and A2 clones exhibited a more intermediate phenotype when grown in IL-3 (i.e. conditions of "self-renewal"), including an increase in cytoplasmic volume, nuclear volume, and cytoplasmic granularity. This was most striking for the A2 clone, which only activates and translocates the mutant full-length Stat5 into the nucleus (see Fig. 11C). The morphology of these cells did not change after culture in GM-CSF. Similar observations have been made with clones expressing an epitope-tagged version of Stat5<sup>b/wt</sup>. These observations provide further evidence that the activity of the Stat5 protease is important for the normal growth of myeloid progenitors.

**DISCUSSION**

Stat5 is encoded for by two highly homologous genes (Stat5a and Stat5b) and transduces signals for the IL-3 family of ligands (1, 2). The proteins encoded by these genes differ only in their carboxyl-terminal transcriptional activation domain (1–3). Stat5a and Stat5b isoforms that are missing this carboxyl terminus have also been identified in several immature myeloid lineages (1, 19, 27, 39–41). Moreover, several groups have reported that a loss in the expression and activation of these isoforms correlates with maturation (1, 39–41). These studies have also determined that truncated Stat5 is generated through a unique protein-processing event (19, 41). In addition, the Stat5 protease has been found to be constitutively activated and associated with the nucleus (19, 41). Consistent with our most recent observations, others have determined that this protease is sensitive to PMSF and cleaves both inactive (i.e. unphosphorylated) and active (i.e. tyrosine-phosphorylated) isoforms of Stat5 (41). These observations suggest that immu-

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2 F. Piazza, J. Valens, and C. Schindler, unpublished observations.
ture myelocytes express a protease that alters the biological response to Stat5-activating ligands. Concordant with this hypothesis, immature myeloid cells exhibit a distinct response to the IL-3 family of ligands (i.e. proliferation and maturation versus the terminal differentiation and activation found in mature cells). Although Stat5 also transduces signals for members of the IL-2 family that are important for lymphoid maturation and activation, there is no evidence that the truncated Stat5 isoforms play a role in this lineage.

Biochemical characterization of the Stat5 protease has highlighted a number of unique features of this protein. First, purification through four sequential steps has yielded an active fraction with a single protein of ~25 kDa. This is consistent with results from our size exclusion chromatography studies, indicating the protease is between 10 and 40 kDa. Moreover, a faint ~25-kDa band was recovered from the most active fractions of this column. Although 25 kDa is small for a protease, a number of small proteases have been identified. This is especially the case in viruses where their small genomes restrict the size of gene products. The smallest viral protease, ~100 amino acids, functions as a dimer, setting a lower limit of ~200 amino acids (55–57). Consistent with this, the smallest cellular serine proteases usually just exceed 200 amino acids (e.g. 25–30 kDa (58–60)). Additionally, sequence comparison between related proteases often identify a conserved region of 200–250 amino acids (58). A second feature, gleaned from purified preparations of the Stat5 protease, is that it is active over a wide range of physiological pH values. A third feature is that the protease exhibits a highly restricted and unique pattern of sensitivity to protease inhibitors. This pattern indicates that the protease is an unusual member of the serine family of proteases. A fourth feature is that the protease cleaves both inactive and active isoforms of Stat5, suggesting it may function constitutively, i.e. cleave Stat5 in unstimulated cells. Whereas the identification of both full-length and truncated isoforms in unstimulated FdTrk and DA-3 cells supports this observation (19), recently published studies suggest that the protease is associated with the nucleus (41). Such a sub-localization could effectively restrict access of the protease to activated Stat5. These possibilities are currently under investigation.

Purified preparations of the protease were also employed to map the site at which Stat5 is cleaved. Mass spectrometry studies clearly placed the cleavage site between Tyr-719 and Met-720 in Stat5a and Tyr-724 and Met-725 in Stat5b. This was confirmed by mutating the two residues flanking the cleavage site and demonstrating that this Stat5b is resistant to cleavage. The biochemical features of this mutant (i.e. its ability to become activated, dimerize, and bind DNA and its companion protein) of both full-length and truncated isoforms in unstimulated FdTrk and DA-3 cells leads to the translocation of activated full-length Stat5 to the nucleus. This corresponds with an important morphological change in these transfectants, that is cells expressing significant levels of non-cleavable Stat5 exhibit a partially differentiated phenotype. These studies indicate that regulation of Stat5 activity is important for normal myeloid development.

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