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Functionally Distinct Isoforms of STAT5 Are Generated by Protein Processing

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Summary

The interleukin-3 family of cytokines, which play an important role in the development of myeloid lineages, transduce signals through the JAK–STAT pathway. Previous studies demonstrate that this process entails the activation of four distinct isoforms of STAT5, where two shorter isoforms are activated in a distinct population of cells. We now demonstrate that the shorter isoforms represent carboxy-terminal truncations. Moreover, these truncations are not generated by RNA processing, but by a specific proteolytic activity. Consistent with the notion that truncated STAT5 isoforms transduce distinct signals, they fail to promote the activation of several known interleukin-3 target genes. These studies suggest that the activity of a specific protease may play a critical role in defining the biological responses transduced by STAT5.

Introduction

The ability of the JAK–STAT pathway to transduce high-fidelity signals in response to cytokines was first elucidated for the interferons (Schindler et al., 1992; Schindler and Darnell, 1995). JAKs are receptor-associated tyrosine kinases, and STATs (signal transducers and activators of transcription) are cytoplasmic transcription factors that are activated by JAKs. Studies on other cytokines, including members of the interleukin-3 (IL-3) family (IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor [GM-CSF] [Goodall et al., 1993; Miyajima et al., 1993]) have led to the identification of additional JAKs and STATs (Schindler and Darnell, 1995). These STATs encode a number of highly conserved domains that contribute to the transduction of ligand-specific signals (Schindler and Darnell, 1995; Gupta et al., 1996). In contrast, the divergent carboxy termini appear to encode a transcription activation domain (Müller et al., 1993; Bhattacharya et al., 1996; Mui et al., 1996; Qureshi et al., 1996). Consistent with these observations, functionally distinct carboxy-terminally truncated STAT isoforms have been reported. For example, naturally occurring truncated STAT1 and STAT3 bind DNA but fail to activate transcription as independent factors (Müller et al., 1993; Caldenhoven et al., 1996).

The IL-3 family of cytokines have been shown to play an important role in the maturation, proliferation, and activation of myeloid lineages. Of this group, the spectrum of activity of IL-5 appears to be predominantly restricted to the development and then subsequent activation of eosinophils, basophils, and mast cells. In contrast, IL-3 and GM-CSF have significantly broader and overlapping roles in the growth, differentiation, and activation of multiple myeloid lineages (Sachs, 1993). Consistent with their overlapping biological functions, these cytokines bind a family of closely related receptors (Goodall et al., 1993; Kitamura et al., 1994). Each receptor consists of a unique ligand-binding α chain and a common signal-transducing β chain. Once bound by ligand, this family induces the expression of genes through the sequential activation of JAK2 and STAT5 (Azam et al., 1995; Mui et al., 1995; Quelle et al., 1994). Upon activation, STAT5 is released from the receptor, dimerizes, translocates to the nucleus, and binds to a member of the γ activation site (GAS) family of enhancers. This in turn culminates in the activation of target genes (Schindler and Darnell, 1995). Uniquely, the IL-3 signaling paradigm employs four distinct STAT5 isoforms, which are encoded for by the highly homologous *Stat5a* and *Stat5b* genes (Azam et al., 1995; Liu et al., 1995; Mui et al., 1995). Each gene gives rise to both a long and a short isoform. Previous studies have shown that the short and long isoforms are activated in distinct populations of cells (Rothman et al., 1994; Azam et al., 1995).

In the current set of studies we demonstrate that the short isoforms are missing the 70- to 80-most carboxy-terminal amino acids, which have been reported to encode the transcriptional activation domain of STAT5 (Moriggl et al., 1996; Mui et al., 1996). Consistent with this observation, cells in which the truncated isoforms of STAT5 are activated fail to induce several IL-3 target genes. Moreover, these truncated isoforms appear to be generated by a STAT5-cleaving protease, the activity of which is limited to a distinct population of cells. These observations suggest that the generation and subsequent activation of carboxy-terminally truncated isoforms of STAT5 may account for the phenotypically distinct responses some cells exhibit to members of the IL-3 family of ligands.

Results

Structural Analysis of the Truncated Isoforms of STAT5

We have previously described two types of DNA-binding complexes induced by members of the IL-3 family of cytokines (Azam et al., 1995; Rothman et al., 1994). In two cell lines that appear to represent an earlier stage of myeloid development, DA-3 and FDC-P1 (Miura et al., 1991; Bourette et al., 1995), a distinct, faster-migrating DNA-binding complex, STF-IL3a, is activated (Azam et al., 1995). STF-IL3a is also activated in another myeloid cell line, 32Dc1, after stimulation with IL-3 (Figure 1, lane 4). Moreover, a differentiated subline of 32D that exhibits early erythroid features (Migliaccio et al., 1989) activates a different and more slowly migrating complex in response to IL-3 or erythropoietin (Rothman et al., 1994).

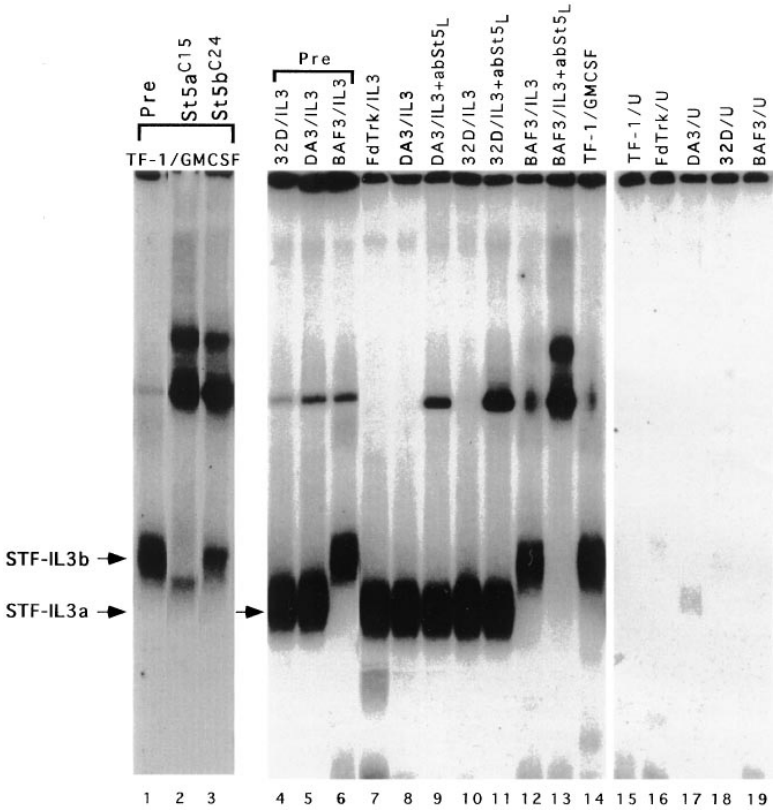

Figure 1. The Components of STF-IL3a Are Not Recognized by Carboxy-Terminal STAT5a- and STAT5b-Specific Antibodies

Nuclear extracts, prepared from GM-CSF–stimulated TF-1 cells and IL-3–stimulated 32Dc1, DA-3, Ba/F3, and FdTrk cells were incubated with antisera and evaluated by EMSA with an *IRF-1* GAS probe. The extracts were either incubated in nonimmune serum (Pre; lanes 1 and 4–6), St5a^{C15} (lane 2), St5b^{C24} (lane 3), or a combination of St5a^{C15} and St5b^{C24} (STAT5$_L$; lanes 9, 11, and 13). Extracts prepared from unstimulated (U) cells (lanes 15–19) are shown as controls. The mobility of STF-IL3a and STF-IL3b is indicated in the left margin.

This complex is referred to as STF-IL3b and has been shown to be activated by IL-3, IL-5, and GM-CSF in a number of established myeloid cell lines with erythroid, monocytic, or mast cell–like features (Azam et al., 1995; Mui et al., 1995; Pallard et al., 1995). To determine whether these differing complexes might be responsible for the distinct pattern of responses observed in some of these different populations of cells, we set out to identify their components.

In a previous set of studies we established, through purification, that STF-IL3a consisted of 77 and 80 kDa isoforms of STAT5a and STAT5b respectively (Azam et al., 1995). However, because the STAT5a and STAT5b cDNAs appeared to encode proteins larger than 77 and 80 kDa (Azam et al., 1995; Mui et al., 1995) and STF-IL3b consisted of immunologically related 94 and 96 kDa proteins, we speculated that the *Stat5* genes each gave rise to a full-length and a shorter isoform (Azam et al., 1995). To evaluate the structural relationship of these isoforms, specific antibody and cDNA reagents were prepared. As shown in Figure 1, antibodies directed against the carboxy terminus encoded for by both STAT5a and STAT5b cDNAs recognize distinct components of the cluster of three bands that constitute STF-IL3b (the GM-CSF–stimulated TF-1 cells extracts shown in lanes 1–3 [Azam et al., 1995]). The STAT5a antibody recognizes proteins in the two slower complexes, whereas the STAT5b antibody recognizes proteins in the two faster complexes. These observations are consistent with transfection studies indicating that the STAT5b homodimer has a faster mobility than the STAT5a homodimer and that the STAT5a–STAT5b heterodimer has an intermediate mobility (Figure 2C).

Similar studies were carried out to evaluate DNA-binding complexes induced by IL-3 in several murine cell lines, employing a pooled set of the STAT5a and STAT5b antibodies (Figure 1). These cell lines included 32Dc1 (Migliaccio et al., 1989), DA-3 (Miura et al., 1991), FdTrk (a derivative of FDC-P1 cells [Isfort et al., 1988; Azam et al., 1995]), and Ba/F3 (Daley and Baltimore, 1988) cells. Consistent with the studies in GM-CSF–stimulated TF-1 cells, these antibodies effectively "supershift" (i.e., recognize) the components of the STF-IL3b complex in IL-3–stimulated Ba/F3 cells (lanes 12–14). However, they fail to interact with components of STF-IL3a complexes in DA-3 and 32Dc1 cells (see lanes 8–11). Antibodies that recognize more amino-terminal domains recognize proteins in both complexes (data not shown; Azam et al., 1995). These observations indicate that the carboxy terminus of STAT5a and STAT5b, present in the 94 and 96 kDa components of STF-IL3b, are missing from the 77 and 80 kDa isoforms of STAT5a and STAT5b.

To confirm these supershifting results, full-length and carboxy- terminally truncated *Stat5a* and *Stat5b* cDNA expression constructs were prepared (Figure 3A). *Stat5a*$_t$ and *Stat5b*$_t$ were designed to correspond to two recently described rat cDNAs that encode putative STAT5 isoforms truncated at amino acids 740 (STAT5a$_t$ [Kazansky et al., 1995]) and 745 (STAT5b$_t$ [Ripperger et al., 1995]), respectively. An additional smaller truncation, giving rise to a 714 amino acid STAT5b protein, was generated by introduction of a stop codon at a convenient NsiI endonuclease restriction site (*Stat5b*$_{Nsi}$). The

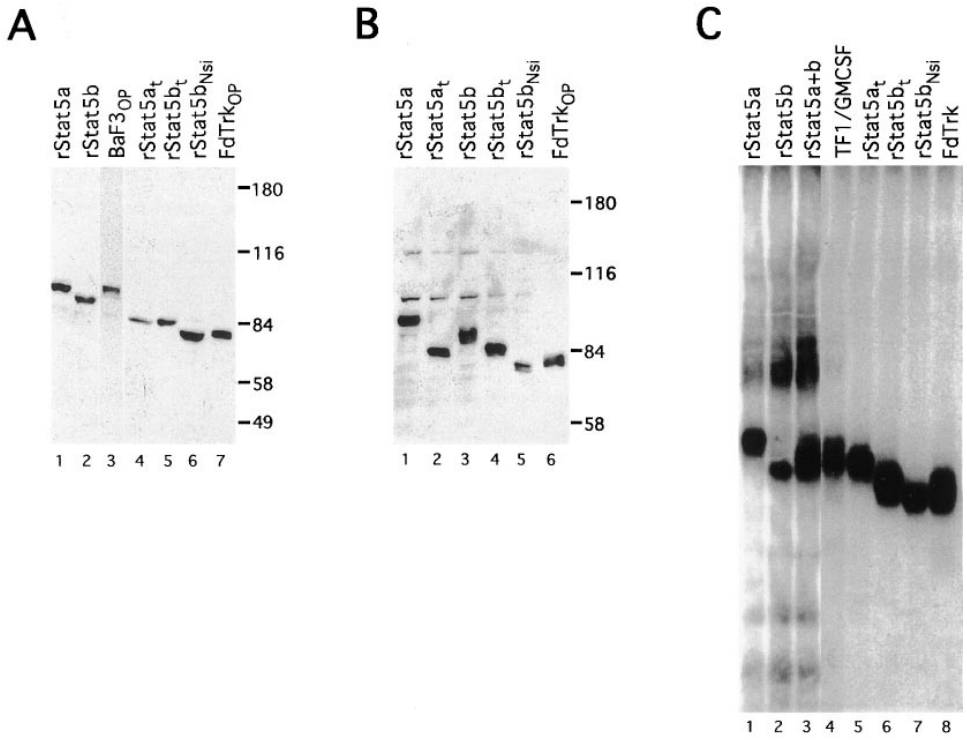

Figure 2. Characterization of Truncated Isoforms of STAT5

(A) Western blot of a panel of recombinant unphosphorylated STAT5 isoforms. Extracts prepared from 293 cells transiently transfected with full-length STAT5a (lane 1), full-length STAT5b (lane 2), STAT5a truncated at amino acid 740 (STAT5a$_t$, lane 4), STAT5b truncated at amino acid 745 (STAT5b$_t$, lane 5), or STAT5b truncated at amino acid 714 (STAT5b$_{Nsi}$, lane 6) were fractionated by SDS-PAGE and immunoblotted by a pan-STAT5 antibody (Transduction Laboratory). Oligonucleotide precipitations (Azam et al., 1995) of extracts prepared form IL-3–stimulated Ba/F3 (lane 3) or FdTrk (lane 7) cells are included as controls. Molecular weight markers (in kilodaltons) are indicated in the right margin.

(B) Western blot of a panel of recombinant tyrosine phosphorylated STAT5 isoforms. Extracts prepared from 293 cells transiently transfected with full-length STAT5a (lane 1), STAT5a truncated at amino acid 740 (STAT5a$_t$, lane 2), full-length STAT5b (lane 3), STAT5b truncated at amino acid 745 (STAT5b$_t$, lane 4), or STAT5b truncated at amino acid 713 (STAT5b$_{Nsi}$, lane 5) were activated by stimulation of a cotransfected erythropoietin receptor (Klingmueller et al., 1995), fractionated by SDS-PAGE, and immunoblotted with a pan-STAT5 antibody (Transduction Laboratories). Oligonucleotide precipitations (Azam et al., 1995) of extracts prepared from IL-3–stimulated FdTrk cells are included as a control for p77 and p80 (lane 6). Molecular weight markers (in kDa) are indicated in the right margin.

(C) EMSA of the recombinant tyrosine phosphorylated STAT5 isoforms. Whole-cell extracts of the transfectants described in (B) were evaluated by EMSA with an *IRF-1* GAS probe. In lane 3, equal volumes of recombinant STAT5a and STAT5b were coincubated with the GAS probe. Controls include GM-CSF–stimulated TF-1 cells (lane 4) and IL-3–stimulated FdTrk cells (lane 5). Lanes 1–3 represent an approximate 3× exposure compared to lanes 4–8.

corresponding recombinant proteins were expressed in 293 cells and evaluated by immunoblotting. As had been previously reported, the full-length Stat5a and Stat5b cDNAs gave rise to the predicted 96 and 94 kDa isoforms (Figure 2A, lanes 1 and 2). Both *Stat5a$_t$* and *Stat5b$_t$* encoded proteins that are larger, and *Stat5b$_{Nsi}$* encoded one that is smaller in apparent molecular weight than p77 and p80 (Figure 2A, lanes 4–7).

Since tyrosine phosphorylation can alter the relative migration of proteins in SDS polyacrylamide gel electrophoresis (PAGE) and our preparations of p77 and p80 are tyrosine phosphorylated, the experiment was repeated after activation of each of the truncated STAT5 isoforms through the stimulation of a cotransfected receptor. Again, both of the "rat-like" isoforms migrated more slowly and the Nsi isoform faster than bona fide p77 and p80 (Figure 2B). The same activated recombinant STAT5 isoforms were evaluated by electrophoretic mobility shift assay (EMSA) to confirm that they had been activated and to evaluate their relative mobility (Figure 2C). Consistent with the SDS-PAGE analysis, the rat-like isoforms migrated more slowly than native p77/p80 (STF-IL3a), whereas the complex generated by the Nsi truncation migrated more rapidly. These studies not only provide convincing evidence that the rat-like alternative RNA products do not give rise to the 77 and 80 kDa murine isoforms of STAT5 but also confirm that STF-IL3b consists of full-length isoforms of STAT5a and STAT5b (Figure 2C, lanes 1–3). In addition, these studies suggest that the truncation that gives rise to p80 must occur between amino acids 714 and 745 of STAT5b and, by inference, between amino acids 709 and 740 of STAT5a.

Are the Carboxy-Terminally Truncated Isoforms Generated by RNA Processing?

The preceding structural studies not only determined that p77 and p80 represent carboxy-terminally truncated isoforms of STAT5a and STAT5b but suggested that they are not generated through the RNA-processing

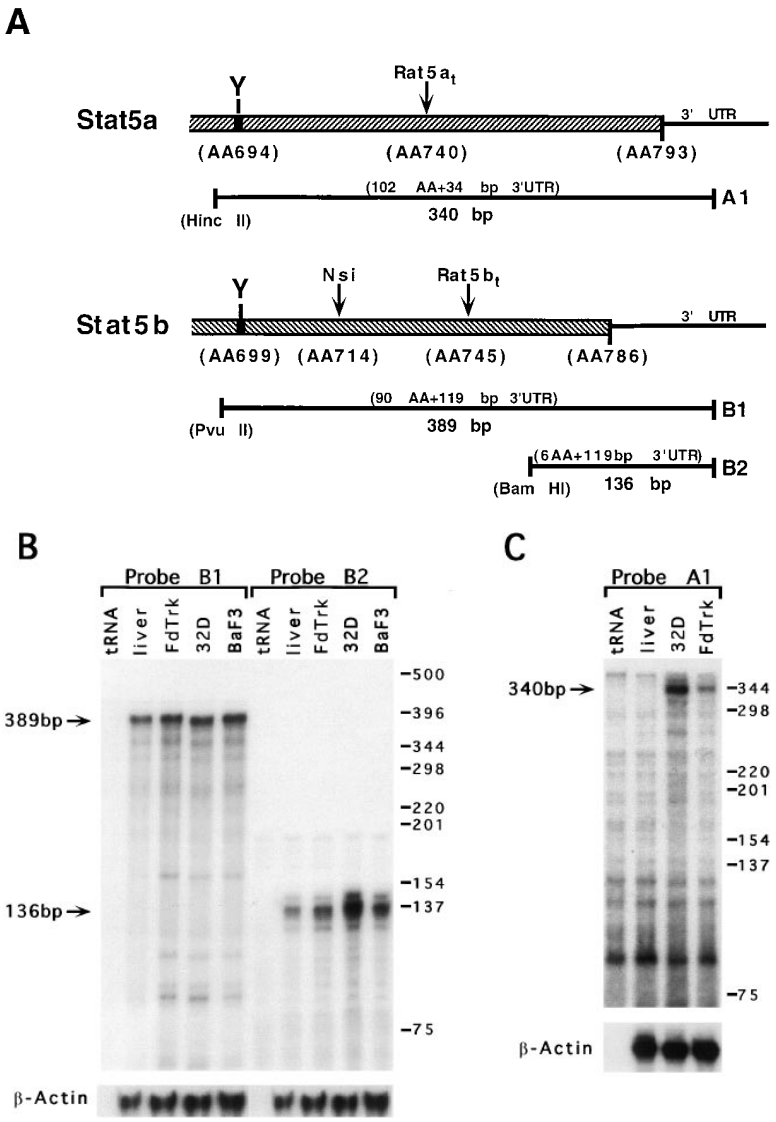

Figure 3. Evaluation of Alternative *Stat5* Transcripts by an RNAse Protection Assay

(A) Schematic representation of the carboxy terminus of STAT5a and STAT5b. The RNAse protection probes for *Stat5a* (A1) and for *Stat5b* (B1 and B2) are indicated. Tyrosine phosphorylation sites (Y), stop codons, and the 3′ untranslated region (3′ UTR) of *Stat5a* and *Stat5b* are indicated. Hatched bars represent the coding regions of *Stat5a* and *Stat5b*. *Rat5a*$_t$, *Rat5b*$_t$, and *Nsi* represent carboxy-terminal truncations. Probes were prepared by subcloning the illustrated fragments with the 5′ restriction site in the coding sequence as indicated and a 3′ site in the poly linker.

(B) RNAse protection assay with two *Stat5b* probes. Ten micrograms of total RNA, prepared from murine liver, 32D cells, FdTrk cells, Ba/F3 cells, or tRNA was incubated with riboprobes B1 and B2; digested with RNAse T2; and fractionated on a standard sequencing gel. A β-actin probe served as a loading control. Molecular weight markers (in base pairs) and the length of the fully protected B1 and B2 probes are indicated in the right and left margins, respectively.

(C) RNAse protection assay with a *Stat5a* probe. Ten micrograms of total RNA from liver and 32D cells, FdTrk cells, or tRNA was evaluated as described for (B) with probe A1. Again, a β-actin probe served as a loading control. Molecular weight markers (in base pairs) and the length of the fully protected A1 probe are indicated in the right and left margins respectively.

event recently reported in another rodent species (Kazansky et al., 1995; Ripperger et al., 1995). However, because studies on the generation of the truncated isoforms of STAT1 and STAT3 had established a precedent for RNA processing (Müller et al., 1993; Schaefer et al., 1995; Caldenhoven et al., 1996), this possibility was examined more rigorously, through a set of RNAse protection assays. Riboprobes spanning the appropriate carboxy-terminal regions of murine STAT5a and STAT5b were prepared (Figure 3A). One additional carboxy-terminal *Stat5b* riboprobe, B2, was designed to hybridize only to transcripts encoding the full-length isoform of STAT5b. These riboprobes were incubated with RNA prepared from either murine liver or IL-3–responsive cells, digested with RNAse T2, and evaluated on a denaturing gel. As expected, the B1 probe was fully protected when incubated with RNA prepared from Ba/F3 cells but was fully digested when incubated with tRNA (Figure 3B). Probe B1 (and B2), but not A1, were also fully protected in liver, corresponding with previous reports indicating that STAT5b is the predominant isoform expressed in liver (Wakao et al., 1994; Ripperger et al., 1995). Intriguingly, both *Stat5b* probes were fully protected when incubated with RNA prepared from cells in which the truncated isoform of STAT5b is found (FdTrk and 32Dc1 cells; see Figs. 3B). Similar results were obtained with the *Stat5a* probe, excluding the existence of all but trace amounts of alternative RNA splice products in these cells. Furthermore, RNAse protections studies with the more 3′ B2 probe indicated that *Stat5b* full-length transcript is expressed at equivalent levels in each of these cell types.

Are the Carboxy-Terminally Truncated Isoforms Generated by Protein Processing?

The lack of evidence for RNA species that could give rise to p77 or p80 provided indirect support for a protein-processing model. In this model, a specific protease would process p96 and p94 into p77 and p80, respectively. To evaluate this model, we first needed to establish, as predicted by the RNAse protection studies, that full-length proteins (p94 and p96) were synthesized in cells in which p77 and p80 are activated. To this end,

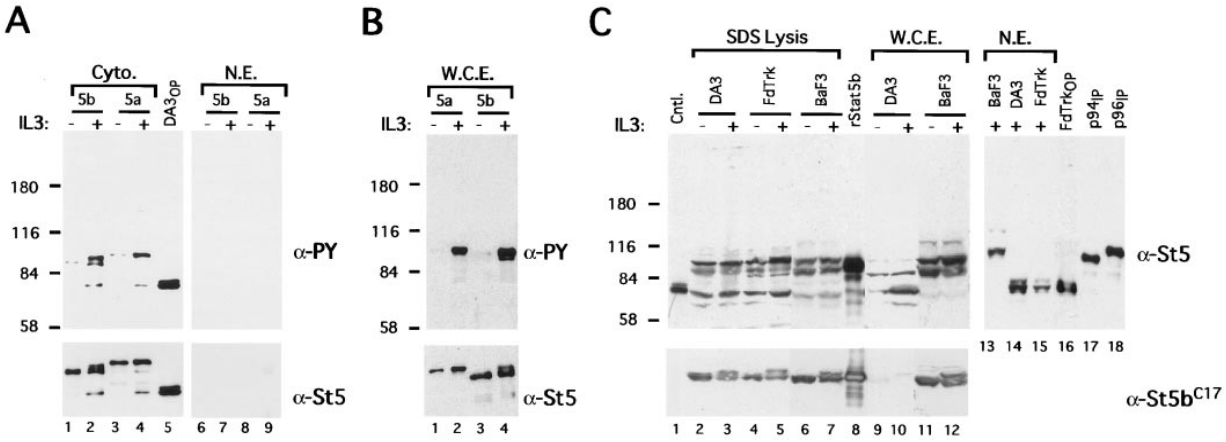

Figure 4. Differential Expression of STAT5 Isoforms in Several Cell Types

(A) Immunoprecipitation of full-length STAT5 isoforms from DA-3 cells. Cytoplasmic (Cyto.) and nuclear extracts (N.E.), prepared from unstimulated (−) and IL-3–stimulated (+) DA-3 cells were immunoprecipitated with carboxy-terminal STAT5a (5a [St5a^{C15}]) and STAT5b (5b [St5b^{C24}]) antibodies, fractionated by SDS-PAGE, and sequentially immunoblotted with antiphosphotyrosine (α-PY, Upstate Biologicals) and pan-STAT5 (α-St5, Transduction Laboratories) antibodies (lane 1–8). Oligonucleotide precipitation of p77 and p80 for a matched set of nuclear IL-3–stimulated DA-3 extracts are shown as a control (lane 9). Molecular weight markers (in kilodaltons) are indicated in the left margin.

(B) Immunoprecipitation of full-length STAT5 isoforms from Ba/F3 cells. Whole cell extracts (W.C.E), prepared from unstimulated (−) and IL-3–stimulated (+) Ba/F3 cells, were immunoprecipitated with carboxy-terminal STAT5a (5a [St5a^{C15}]) and STAT5b (5b [St5b^{C24}]) antibodies; fractionated by SDS-PAGE; and sequentially immunoblotted with antiphosphotyrosine and pan-STAT5 antibodies as in (A) (lane 1–4). Molecular weight markers (in kilodaltons) are indicated in the left margin.

(C) Western blot of native STAT5 isoforms expressed in IL-3–responsive cells. Extracts were prepared from matched sets of unstimulated (-) and IL-3–stimulated (+) DA-3, FdTrk, and Ba/F3 cells either by rapidly lysing the cells in hot SDS-PAGE sample loading buffer (SDS lysis, lanes 2–7) or by conventional protocols (W.C.E., lanes 9–12; or N.E., lanes 13–15). These extracts were fractionated by SDS-PAGE and sequentially immunoblotted with a pan-STAT5 (α-St5) antibody (lanes 1–18) and St5b^{C17} (lanes 1–12; Santa Cruz Biotechnology). Controls are included in lane 1, p77 and p80 from nuclear FdTrk extracts; lane 8, recombinant STAT5b (see legend for Figure 5); lane 16, oligonucleotide precipitated IL-3–stimulated FdTrk extracts; and p94 (lane 17) and p96 (lane 18) prepared from unstimulated YT cells. Molecular weight markers (in kilodaltons) are indicated in the left margin.

cytoplasmic and nuclear extracts, prepared from DA-3 cells before and after IL-3 stimulation, were immunoprecipitated with the STAT5a and STAT5b carboxy-terminal–specific antibodies (Figure 4A). As a control, the 77 and 80 kDa STAT5 isoforms were collected from a parallel aliquot of IL-3–stimulated extracts by oligonucleotide precipitation (Figure 4A, lane 5). Further controls were provided by a matched set of immunoprecipitations from Ba/F3 cells, where p94 and p96 are activated in response to IL-3 (Figure 4B). Both sets of immunoprecipitates were fractionated by SDS-PAGE and then sequentially immunoblotted with antiphosphotyrosine and a pan-STAT5 antibody. As anticipated, p94 and p96 were tyrosine phosphorylated in stimulated but not unstimulated Ba/F3 cells (Figure 4B). Reprobing with the pan-STAT5 antibody confirmed that the tyrosine-phosphorylated and unphosphorylated isoforms of STAT5a (p96) essentially comigrate (Figure 4B, lanes 1 and 2), whereas the tyrosine-phosphorylated isoform of STAT5b (p94) migrates more slowly than the unphosphorylated form (Figure 4B, lanes 3 and 4 [Mui et al., 1995]). The detection of both STAT5b isoforms in stimulated Ba/F3 cells indicates that only about one half of STAT5b is activated after 15 min of IL-3 stimulation (Figure 4B, lane 4).

Evaluation of the extracts prepared from DA-3 cells provides an important contrast to the Ba/F3 cells (Figure 4A). As predicted by the protein-processing model, p94 and p96 are abundantly expressed in unstimulated DA-3 cells. Consistent with previous studies, p77 and p80 become rapidly activated after stimulation with IL-3 in these cells (Figure 4A, lane 5). However, with the aid of p94- and p96-specific antibodies it is possible to demonstrate that some of the full-length isoforms become activated early after stimulation with IL-3 (Figure 4A, lanes 2 and 4). The recovery of p77 in the STAT5a and STAT5b immunoprecipitations suggests that activated p77 may associate with the full-length isoforms in the cytoplasm. A more quantitative EMSA study (data not shown) confirms the relatively modest (in comparison to p77 and p80) activation of p94 and p96 in cytosolic DA-3 extracts prepared after 5 to 15 min of stimulation with IL-3 but not after longer periods of stimulation. However, in contrast to what is observed in the control Ba/F3 cells, the p94 and p96 activated in DA-3 cells fail to translocate to the nucleus (Figure 4A, lanes 7 and 9; Figure 4C, lanes 14 and 15). Only truncated p77 and p80 are found in these nuclei (Figure 1, lanes 7–11; Figure 4C [Rothman et al., 1994; Azam et al., 1995]). These observations not only provide further support for the protein processing model; they also suggest that only the truncated isoforms have access to the nucleus in these cells.

Next we wanted to determine whether STAT5 processing was a constitutive or dynamic (ligand dependent). The pattern of STAT5 expression was evaluated by immunoblotting samples prepared from DA-3, FdTrk, and Ba/F3 cells either by rapid lysis/denaturation or by more standard protocols (e.g., whole-cell extracts

[Rothman et al., 1994] or nuclear extracts [Dignam et al., 1983]). The nuclear and whole-cell extracts from IL-3–stimulated DA-3 (and FdTrk) cells confirm that truncated STAT5 is the major isoform recovered from these compartments, consistent with a more dynamic process (Figure 4C, lanes 10, 14 and 15). Note that p77 (STAT5a) appears to be more the abundant isoform in these cells. Parallel studies with nuclear and whole-cell extracts from Ba/F3 cells indicate that only full-length isoforms of STAT5 are recovered from these compartments (Figure 4C, lanes 11–13). Again, STAT5a (p96) appears to be more abundant. Extracts prepared by the SDS lysis protocol support a more static process. As anticipated, p94 and p96 are readily detected in each of the unstimulated cell types (Figure 4C, lanes 1–6). Truncated STAT5 isoforms are consistently detected in starved/unstimulated DA-3 and FdTrk cells (Figure 4C, lanes 2 and 4), suggesting either that truncated STAT5 isoforms have a long half-life (i.e., survive through the starvation period), or that these isoforms are generated constitutively. Ligand induced changes are relatively subtle for all three cell types. When DA-3 (factor dependent) and FdTrk (factor independent) cells are stimulated, there appears to be a modest loss of p94 and a corresponding modest increase in p77 (Figure 4C, lanes 3 and 5). Consistent with the immunoprecipitation studies (Figure 4A), reprobing with a STAT5b-specific antibody (α-St5^{C17}) indicates that some of the p94 loss can be attributed to tyrosine phosphorylation (i.e., an upward shift in mobility). Similarly, in Ba/F3 cells there is only modest evidence for IL-3 dependent activation of p94 (i.e., an upward shift in mobility) in SDS lysis samples, despite clear evidence for this in nuclear extracts (Figure 1 and Figure 4C, lanes 10–11). Although these observations are unable to distinguish clearly between a static or dynamic model of STAT5 processing, they seem to suggest that only the appropriate isoforms localize to a compartment(s) that is permissive for nuclear translocation. This will need to be confirmed by further studies. In addition, these observations indicate that protein processing is essentially restricted to DA-3 and FdTrk cells.

Characterization of STAT5 Protease Activity

To find more direct evidence for a protease, a STAT5-cleaving assay was established. This entailed the preparation of recombinant STAT5b immobilized on an oligonucleotide affinity matrix. As shown in Figure 5A, when the STAT5b is eluted from this matrix and fractionated on SDS-PAGE, the expected major band of 94 kDa is detected with the STAT5 monoclonal antibody. Consistent with the protease model, when this preparation of STAT5b is incubated with a CHAPS extract prepared from Ba/F3 cells, only full-length p94 is recovered. However, when STAT5b is incubated with a CHAPS extract prepared from FdTrk cells (or DA-3 cells; data not shown), it is processed to the appropriate 80 kDa isoform. Moreover, this cleavage is fully inhibited by the

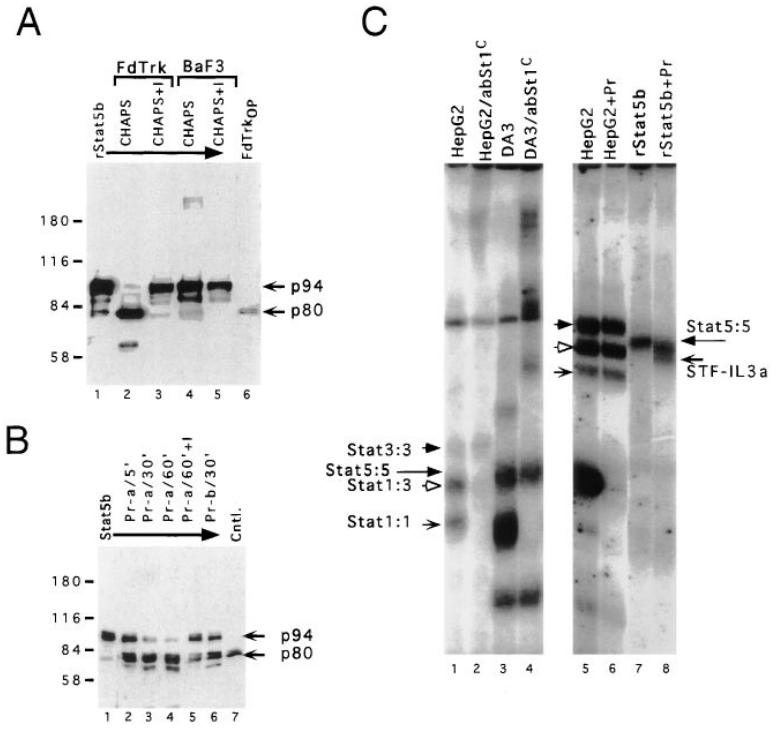

Figure 5. STAT5-Cleaving Activity Is Present in Extracts Prepared from FdTrk but Not Ba/F3 Cells

(A) Recombinant tyrosine-phosphorylated STAT5b, prepared from insect cells, was collected by oligonucleotide precipitation (lane 1), and incubated (1 hr at 37°C) with CHAPS extracts prepared from FdTrk (lane 2 and 3) or Ba/F3 (lane 4 and 5) cells in the presence or absence of PMSF as indicated. Proteins were recovered by heating in SDS-PAGE sample buffer, resolved by SDS-PAGE, and immunoblotted with a pan-STAT5 antibody (Transduction Laboratories). Molecular weight markers (in kDa) and the mobility of both isoforms of STAT5b (p94 and p80) are indicated in the left and right margins, respectively.

(B) Partially purified preparations of protease were assay as described in (A). Three microliters of heparin agarose–fractionated protease activity (Pr-a) was incubated with 5–10 μl immobilized STAT5b for 5, 30, and 60 min (lanes 1–5) at 37°C. PMSF (I) was added in lane 5. In lane 6, a less potent preparation of protease (Pr-b) was assayed for 30 min. Controls include lane 1, uncut STAT5b; and lane 7, IL-3–stimulated FdTrk nuclear extract. Molecular weight markers (in kilodaltons) and the mobility of both isoforms of STAT5b (p94 and p80) are indicated in the left and right margins, respectively.

(C) EMSA to evaluate the specificity of the protease. Extracts were prepared and assayed as described in legend to Figure 1. Lane 1, IL-6–stimulated HepG2 cells; lane 2, IL-6–stimulated HepG2 cells supershifted with STAT1 carboxy-terminal–specific antibody (Schindler et al., 1992); lane 3, murine IFNγ-stimulated DA-3 cells; lane 4, murine IFNγ-stimulated DA-3 cells supershifted with STAT1 carboxy-terminal–specific antibody; lane 5, 1 μl of IL-6–stimulated HepG2 cells; lane 6, IL-6–stimulated HepG2 cells incubated with 3 ml of heparin agarose fractionated protease at 37°C for 1 hr prior to EMSA; lane 7, 1 μl recombinant STAT5b; and lane 8, recombinant STAT5b incubated with 3 μl of heparin agarose–fractionated protease at 37°C for 1 hr prior to EMSA.

serine protease inhibitor phenyl-methyl sulfonyl fluoride (PMSF). Similar studies with partially purified preparations of the protease confirm the sensitivity to PMSF (Figure 5B, lane 5) and provide further evidence for the protease by demonstrating that it cleaves its substrate in a time-dependent process (Figure 5B, lanes 1–4). Consistent with these kinetic studies, an equivalent volume of a cruder preparation of protease has less activity at 30 min (Figure 5B, lane 6). Of a cautionary note, even though the major cleavage product with partially purified preparations of protease is bona fide p80, additional smaller bands are generated. We believe that this is due to overdigestion, since these products appear to be generated in a dose-dependent manner and there is no loss of specificity.

Next, both in vivo and in vitro studies were carried out to evaluate the specificity of the protease activity. As shown in Figure 5C (lanes 1–4), when DA-3 cells are stimulated with interferon-γ (IFNγ), the previously characterized doublet of STAT1 DNA-binding activity is observed (Rothman et al., 1994). This complex does not contain any carboxy-terminally truncated STAT1, since it is supershifted as effectively as control STAT1 with a STAT1-, carboxy-terminal–specific antibody. Furthermore, the DNA-binding activities of STAT1 and STAT3 are not affected by incubation with a partially purified preparation of protease (Figure 5C, lanes 5–8). Yet, parallel studies with preparations of full-length STAT5b demonstrate the anticipated protease-dependent conversion to a faster-migrating DNA-binding complex (STF-IL3a). These studies provide compelling evidence for the existence of a serine protease in FdTrk and DA-3 cells (data not shown) that rapidly and specifically cleaves full-length STAT5 into a physiologically relevant truncated isoform.

The Carboxy-Terminally Truncated STAT5 Isoforms Are Functionally Distinct

Studies on STAT1, STAT2, and STAT3 indicate that the carboxy terminus encodes the transcriptional activation domain (Bhattacharya et al., 1996; Caldenhoven et al., 1996; Müller et al., 1993; Qureshi et al., 1996). Moreover, carboxy-terminally truncated isoforms of STAT1 and STAT3, which retain DNA-binding activity, can behave as dominant negative isoforms, suppressing transcription (Müller et al., 1993; Caldenhoven et al., 1996). These observations suggest that the naturally occurring carboxy-terminally truncated STAT5 isoforms might similarly be defective in the activation of target genes. To test this, the expression of several IL-3–stimulated immediate early genes was evaluated. The panel of immediate early genes included both bona fide (e.g., cytokine-inducible SH2-containing protein [*CIS*] and oncostatin M [*OSM*] genes [Yoshimura et al., 1995, 1996]) and potential (e.g., c-*fos*, c-*myc*, and *pim-1* [Mui et al., 1996; Quelle et al., 1996; Wang et al., 1996]) targets of the IL-3–stimulated JAK–STAT pathway. As anticipated, Northern blotting studies revealed that each of these genes is rapidly activated in response to IL-3 in the Ba/F3 cells (Figure 6A). However, the pattern of gene activation was significantly different in FdTrk cells. Only c-*myc* was activated with kinetics similar to the Ba/F3 cells, while the other genes remained quiescent (Figure 6A). Since IL-3 is not an essential growth factor for FdTrk cells and WEHI-conditioned media may contain confounding activities, the experiment was repeated with recombinant IL-3 in the IL-3–dependent cell line DA-3 (Figure 6B). Again, c-*myc*, *CIS*, and *OSM* were rapidly induced in Ba/F3 cells, but *CIS* and *OSM* failed to be induced with the kinetics of an immediate early gene. The modest activation of *CIS* at 180 min is not consistent with activation through the JAK–STAT pathway, but rather some secondary pathway. These observations concur with recently published studies showing that carboxy-terminally truncated STAT5 cDNAs behave as dominant negative isoforms, actively repressing IL-3–stimulated activation of *OSM* and *CIS* (Mui et al., 1996; Wang et al., 1996). These studies suggest that the naturally truncated STAT5 isoforms are functionally distinct and are likely to contribute to a unique biologic response to IL-3 in the cells where they are expressed.

Discussion

Recent studies have determined all three members of the IL-3 family transduce signals through the same JAK–STAT signaling paradigm. In contrast to other cytokines, the IL-3 family signals through distinct subsets of STAT5 isoforms. In some cells the 77 and 80 kDa isoforms of STAT5a and STAT5b are activated and transduce signals to the nucleus. In other cells the 96 and 94 kDa isoforms of STAT5a and STAT5b are activated and transduce signals to the nucleus. Studies in several established hematopoietic murine cell lines suggest that the truncated isoforms may be preferentially activated in more immature lineages (see above [Rothman et al., 1994; Azam et al., 1995; Wang et al., 1996]). However, this has not been determined in primary cells. To evaluate the possibility that the long and short STAT5 isoforms may be functionally distinct, we initially examined their structural relationship. These experiments demonstrated that p77 and p80 represent carboxy-terminal truncations of the full-length 96 and 94 kDa isoforms of STAT5a and STAT5b. Studies in which stop codons were experimentally introduced into the carboxy termini of the *Stat5b* (and *Stat5a*) cDNAs indicate that p80 represents a truncation between amino acids 714 and 745. By analogy, p77 appears to represent a truncation of STAT5a between amino acids 709 and 740 (Figure 3A). Since studies on other STATs have suggested that these deleted regions may encode a transcriptional activation domain (Müller et al., 1993; Bhattacharya et al., 1996; Mikita et al., 1996; Qureshi et al., 1996), we wondered whether this might explain the distinct biological responses exhibited by developmentally related lineages to the same ligand (Sachs, 1993). Consistent with this possibility, we found a direct correlation between the activation of several IL-3 target genes and the presence of a STAT5 carboxy terminus. Cells in which the truncated isoforms of STAT5 are activated fail to rapidly induce most of these genes, including those known to be activated by the JAK2–STAT5 pathway (*CIS* and *OSM* [Yoshimura et al., 1995; Yoshimura et al., 1996]) and several potentially activated by this pathway (*pim-1* and

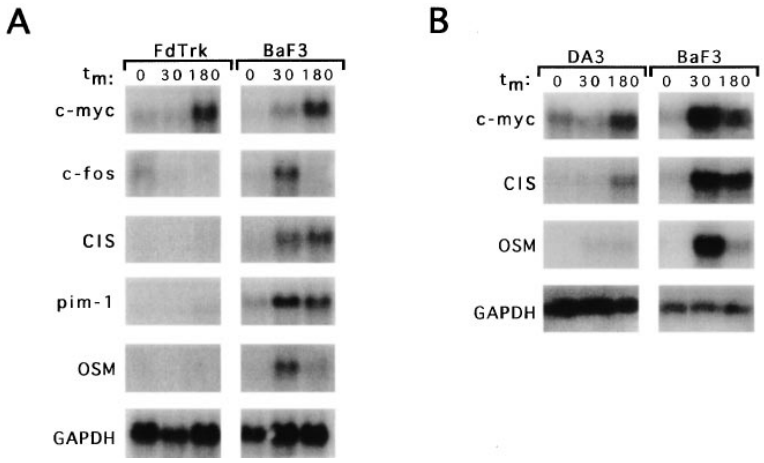

Figure 6. Differential Induction of Immediate Early Genes in Response to IL-3 in Immature and Mature Cells

(A) Ten micrograms of RNA, recovered from FdTrk and Ba/F3 stimulated with 10% WEHI-conditioned medium, for the indicated periods of time (t_m, time in minutes), was fractionated on a formaldehyde gel (Azam et al., 1995), transferred to a nitrocellulose membrane, and sequentially hybridized with radiolabeled c-*myc*, c-*fos*, *CIS*, *pim-1*, *OSM*, and *GAPDH* probes as indicated.

(B) Ten micrograms of of RNA, recovered from DA-3 and Ba/F3 cells stimulated with 10 ng/ml of recombinant IL-3 for the indicated periods of time, was evaluated as described in (A) with c-*myc*, *CIS*, *OSM*, and *GAPDH* probes as indicated.

c-*fos*; Sato et al., 1993; Quelle et al., 1996). c-*myc*, which is induced in both cell types, is likely to be activated by a different more conserved pathway. These functional studies, which assign distinct properties to the truncated STAT5 isoforms, are supported by results from other groups demonstrating that overexpression of truncated STAT5 isoforms can block the ability of the native full-length STAT5 to activate target genes (Mui et al., 1995; Wang et al., 1996).

Previous studies on the carboxy-terminally truncated isoforms of STAT1 and STAT3 have established the precedent that truncated STATs are generated by alternative splicing (Müller et al., 1993; Schaefer et al., 1995; Yan et al., 1995; Caldenhoven et al., 1996). Evidence that the truncated isoforms of STAT5 may also be generated by RNA processing comes from the recent isolation of rat cDNAs encoding isoforms of STAT5 truncated at amino acids 740 (STAT5a) or 745 (STAT5b) (Kazansky et al., 1995; Ripperger et al., 1995). However, our studies indicate that these rat-like *Stat5* cDNAs encode proteins that are too large to be p77 and p80. Furthermore, RNAse protection studies failed to identify any alternative RNA products that might give rise to p77 or p80. Rather, they demonstrated that all cells express the full-length transcripts, even those in which p77 and p80 transduce signals.

In the absence of any evidence for RNA processing, we considered the possibility that the truncated isoforms of STAT5 are generated by a cell-specific protein-processing event. Supporting this model, all cells examined express both full-length *Stat5* transcripts and gene products. A subpopulation of cells express the truncated isoforms of STAT5 as well. Although the existence of both full-length and truncated *Stat5* gene products in FdTrk, DA-3 and 32Dc1 cells does not formally exclude RNA processing, it supports the protein-processing model as well. Further and more compelling support for the protein-processing model comes from the identification of STAT5-specific carboxy-terminal cleaving activity. This activity is found only in cells in which p77 and p80 are expressed and is inhibited by a serine protease inhibitor. Moreover, it exhibits kinetics appropriate for a protease. The identification of this activity in extracts prepared from both unstimulated or stimulated cells (e.g., FdTrk and DA-3) suggests that either this protease is constitutively active or that it resides in a restricted, detergent (CHAPS)–soluble compartment. An intriguing and potentially related observation is that the full-length informs of STAT5 appear to be more difficult to extract from DA-3 and FdTrk cells. These observations and the determination of when the protease is active are currently under investigation. Studies in primary cells await a more definitive identification of this protease.

As these studies were being completed, additional albeit indirect support for RNA-processing model was published (Wang et al., 1996). However, the data in these studies are also consistent with an alternative interpretation. Results from a PCR-based assay, designed to demonstrate the presence of a rat-like RNA "read-through" product in mice, could not distinguish between the possibility of a putative read-through product, a primary transcript, or a DNA template. In addition, an important negative control, RNA prepared from cells expressing full-length isoforms, was not included. The second set of studies, which entailed the characterization of truncated STAT5 isoforms, were intended to corroborate the reverse transcriptase PCR results. However, the truncations these authors introduced were at amino acids 713 (STAT5a) and 718 (STAT5b) and not amino acids 740 and 745 as predicted by their by their RNA read-through model. Hence, their observations support the protein-processing model by demonstrating that the rat-like truncations are too large to encode for p77 and p80.

In summary, our efforts to evaluate the truncated isoforms of STAT5 have determined that they are functionally distinct from the full-length isoforms and that they are generated by a unique protein-processing event. The protease that mediates this is PMSF sensitive, STAT5 specific, and cell type specific. The identification of protease activity in several cell lines that are believed to represent earlier stages in myeloid differentiation (see above) suggests that this protease may be involved in the regulation of lineage-specific STAT5 signaling. Further characterization of this protease should provide the reagents required to explore its activity in vivo.

Experimental Procedures

Cell Culture

Cell culture reagents were purchased from GIBCO–BRL. HepG2, DA-3, 32D, TF-1, WEHI, and FdTrk cells were grown as previously

described (Rothman et al., 1994; Azam et al., 1995; Gupta et al., 1996). Prior to treatment with cytokines, growth factor–dependent cells were starved of these factors and serum for 4–5 hr. TF-1 cells were starved for 16 hr in 0.5% fetal calf serum. HepG2 cells were treated with 200 U/ml of IL-6 (a generous gift from S. Chen-Kiang). DA-3 and 32D and FdTrk cells were treated with recombinant murine IL-3 (10 U/ml, Peprotech) and IFNγ (66 U/ml, Peprotech) or with 10% WEHI-conditioned media. TF-1 cells were stimulated with 50 U/ml GM-CSF (Schering-Plough). 293 cells were grown in Dulbecco's modified Eagle's medium /10% fetal calf serum. For transfection, 293 cells were pretreated with 25 μM chloroquine (Sigma) and then transfected with 5 μg of the STAT5 constructs by the calcium phosphate method (Pear et al., 1993). For STAT activation, 5 μg of an erythropoietin receptor expression construct (a generous gift from H. Lodish [Klingmueller et al., 1995]) or granulocyte colony-stimulating factor (G-CSF) chimeric receptor (I. S. and C. S., unpublished data) were cotransfected and stimulated with either 50 ng/ml of Epo (Amgen) or 500 ng/ml of G-CSF (Amgen) for 30 min.

DNA-Binding Assays and Cell Extracts

The preparation and use of DNA oligonucleotide probes for mobility shift assays has been previously described (Bonni et al., 1993; Pine et al., 1994). Cytoplasmic, nuclear, or whole-cell extracts were made as previously described (Schindler et al., 1992; Eilers et al., 1993; Pine et al., 1994). Next, 0.6 μl of STAT antibodies was added for 60 min (at 4°C) to 1–3 μl of extract prior to a standard shift reaction (e.g., 20 min. at 25°C in 12.5 μl). Oligonucleotide precipitation assays were carried out as previously described (Rothman et al., 1994; Azam et al., 1995). All shift reactions were done with the *IRF-1* GAS probe gatcGATTTCCCCGAAAT (Oligos Etc.).

Antibodies

The pan-STAT5 antibody is a monoclonal antibody purchased from Transduction Laboratories. The STAT5a antibody was raised against the carboxy-terminal 15 amino acids of human STAT5a ($St5a^{C15}$, a generous gift from T. Decker [Meinke et al., 1996]). Antibodies specific for the carboxy terminus of STAT5b were either purchased ($St5b^{C17}$, Santa Cruz Biotechnology) or prepared against a peptide encoding the carboxy-terminal 24 amino acids ($St5b^{C24}$ [Mu et al., 1995]). The antiphosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology.

Northern Blot Analysis and Cloning

Total cellular RNA was isolated by Guanidinium Thiocyanate lysis and cesium chloride gradient centrifugation (Chomczynski and Sacchi, 1987). Ten micrograms of this RNA was then fractionated on 0.8% agarose-formaldehyde gel and transferred to nitrocellulose filters. Filters were hybridized with random prime labeled cDNA probes (Boehringer Mannheim). The c-*myc* probe was a 0.5 kb BamHI–BglII fragment of the cDNA (Wong et al., 1995). The rat c-*fos* probe was a 1.5 kb EcoRI fragment of the cDNA clone *pF222* (Curran et al., 1983). The *CIS* probe was a 250 bp 5′ PCR fragment generated with the primers 5′-AGCACCTACAGAAGATGCCG-3′ (sense) and 5′-ACAAGGCTGACCACATCTGG-3′ (antisense [Yoshimura et al., 1995]). The *OSM* probe was a 500 bp 5′ PCR fragment generated with the primers 5′-ATGCAGACACGGCTTCTAAGAA-3′ (sense) and 5′-GGTGTTGTAGTGGACCGTGAG-3′ (antisense [Yoshimura et al., 1996]). The *pim-1* probe was a 1.0 kb PstI–EcoRI fragment from mouse *pim-1* cDNA (Meeker et al., 1990). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) probe was a 1.2 kb EcoR I fragment from the rat *GAPDH* cDNA clone *pIBI30* (Fort et al., 1985). The riboprobes were generated by [^{32}P]UTP incorporation and transcribing with T3 RNA polymerase (Promega) after linearization of the appropriate pBluescript (Stratagene) constructs with AflIII. $STAT5a_t$ and $STAT5b_t$ expression constructs were generated by the introduction of an appropriately mutated PCR fragment into the NsiI and NotI sites of *Stat5a* (bp 2220–2620) and *Stat5b* (bp 2175–2508). Similarly, the $STAT5b_{Nsi}$ construct was generated by the introduction of a stop codon at the NsiI site in *Stat5b*. The STAT5b baculovirus expression construct was generated by ligating the EcoRI–NotI fragment encompassing the entire open reading frame into the same sites of the pFastBac1 vector (GIBCO/BRL).

Ribonuclease (RNAse) Protection Analysis

RNAse protection studies were carried out as previously described (Melton et al., 1984). In brief, 10 μg of total RNA prepared by guanidinium thiocyanate method was hybridized with A1, B1, and B2 riboprobes ($1–5 \times 10^9$ cpm/μg) in 80% formamide at 48°C. After 16 hr, hybridized products were digested with T2 RNAse (GIBCO/BRL), and protected products resolved on a 5% urea-acrylamide sequencing gel. A 220 bp murine antisense β-actin probe derived from pTRI-β-Actin (Ambion) served as a positive control.

Protein Assays

Proteins were fractionated by SDS-PAGE with a 7% acrylamide resolving gel as previously described (Azam et al., 1995). For immunoblotting, proteins were transferred to nitrocellulose membranes and probed with antibodies at a 1:2000-fold dilution or as recommended by the manufacturer (Pine et al., 1994). An assay for STAT5-cleaving activity was devised by preparing recombinant full-length STAT5b from SF9 cells (Yan et al., 1996). In brief, extracts were prepared by sonication of SF9 cells 4 days after infection. *Stat5b* was collected by oligonucleotide precipitation with a multimerized *IRF-1* GAS element as previously reported (Rothman et al., 1994; Azam et al., 1995). These immobilized preparations of STAT5 were then incubated with DA-3, FdTrk, or Ba/F3 cell extracts prepared by lysis with a CHAPS (3[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate, Sigma)–containing buffer (0.1% CHAPS, 10 mM HEPES [pH 7.4], 2 mM EDTA, 5 mM DTT, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 5% glycerol [Nicholson et al., 1995]). This entailed the addition of 2 volumes of the CHAPS buffer to a pellet of 2×10^6 washed cells by gentle vortexing and a 1 hr 4°C incubation. Subsequently, the lysate were cleared by centrifugation (12,000 × g for 20 min at 4°C). To assay for STAT5-cleaving activity, 20 μl of the cleared lysate was added to 10 μl of immobilized STAT5b beads and incubated for 1 hr at 37°C. STAT5b was recovered by heating in SDS-PAGE sample buffer and then resolving on SDS-PAGE.

Partial purification of this protease (M. A. and C. S., unpublished data) entailed sequential chromatography of FdTrk CHAPS extracts on DEAE Sephacel (Pharmacia) and heparin agarose (Sigma). Protease activity was eluted with a linear 0–1 M NaCl gradient and assayed as outlined above. Fractions with peak activity were pooled and referred to as protease preparation-b (Pr-b) and protease preparation-a (Pr-a).

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