Purification and identification of the STAT5 protease in myeloid cells

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STAT (signal transducer and activator of transcription) proteins are critical regulators of cytokine-induced cell proliferation, differentiation and survival. STAT functional activity can be variably regulated by post-translational modifications, including phosphorylation, acetylation, methylation and sumoylation. Additionally, limited proteolytic digestion of full-length STAT proteins (STAT α) generates C-terminally truncated forms (STAT γ) in different cell lineages, which have significantly reduced transcriptional activity due to the lack of the transactivation domain. Previously, it has been shown that STAT γ , generated by an unidentified nuclear serine protease, plays an important role in myeloid cell differentiation and is aberrantly expressed in acute myeloid leukaemia. To better understand this regulatory mechanism for STAT5 function, we have purified the STAT5 protease from the immature myeloid cell line 32D and identified it by MS analysis as the granule-derived serine protease, CatG (cathepsin

G). We show that purified CatG can specifically cleave full-length STAT5 to generate STAT γ , and this activity can be inhibited by AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] in an *in vitro* protease assay. Importantly, preparation of nuclear and cytoplasmic extracts from immature myeloid cell lines, 32D and FDC-P1, in the presence of a specific inhibitor for CatG results in the identification of STAT α only. These studies indicate that nuclear STAT γ does not naturally exist in immature myeloid cells and is artificially generated from STAT α during the preparation of extracts due to the abundance of CatG in these cells. Therefore in contrast with earlier studies, our data suggest that STAT α , rather than STAT γ is the active form in immature myeloid cells.

Key words: cathepsin G, nuclear, serine protease, STAT5 (signal transducer and activator of transcription 5), truncation.

INTRODUCTION

STAT (signal transducer and activator of transcription) proteins play a major role in cell proliferation, differentiation and survival [1]. Following cytokine or growth factor stimulation, STAT proteins are tyrosine phosphorylated, and the activated dimeric form translocates into the nucleus to induce expression of specific target genes [2–4]. The family of STAT proteins comprises seven members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. There are two homologous STAT5 proteins, STAT5a and STAT5b, which are encoded by two distinct genes [5,6]. Cytokines and growth factors activating STAT5 proteins include those which regulate the proliferation and differentiation of myeloid cells, such as interleukin (IL)-3, IL-5, GM-CSF (granulocyte/macrophage colony-stimulating factor) and thrombopoietin, or differentiation of lymphoid cells, such as IL-2, IL-7 and IL-15 [2]. The important role of STAT5 in the development and survival of haematopoietic cells has been shown in STAT5-deficient mice. Mice deficient in either STAT5a [7] or STAT5b [8] were found to exhibit dramatic defects in non-haematopoietic tissues, but only subtle alterations in the regulation of haematopoietic cells [9–11]. In contrast, mice lacking both STAT5a and STAT5b showed a much stronger defect in the development of myeloid, erythroid and lymphoid lineages [12–14].

In immature myeloid cells, STAT5 has been implicated in both the IL-3-induced proliferation and GM-CSF-induced differentiation of 32D cells [15]. In myeloid progenitors, IL-3 stimulates the activation of C-terminally truncated isoforms of STAT5a (77 kDa) and STAT5b (80 kDa), collectively called STAT γ [16–

21]. In immature myeloid cells the STAT γ isoforms are predominantly localized to the nucleus and activate only a subset of genes activated by STAT α in mature myeloid cells [18,20,22]. It has been demonstrated that STAT γ is generated by limited proteolytic processing of STAT α instead of expression of an alternatively spliced form of STAT5 (STAT5 β) [20]. The protease responsible for the processing of STAT5 was characterized to be a member of the serine protease family, as demonstrated by its sensitivity to the broad-spectrum serine protease inhibitor PMSF. Similar protease activities with the capacity to cleave STAT3 and STAT5 have also been found in acute myeloid leukaemia cells [23] and neutrophils [24–27], and similarly in mast cells to cleave STAT6 [28–30]. Mutation of the mapped cleavage site in STAT5b yielded a more cleavage-resistant mutant STAT5b molecule, which when transfected into the immature myeloid cell line FDC-P1 conferred a strong defect in GM-CSF-induced differentiation of these cells [22,31]. These results suggested that STAT γ is essential for the differentiation of immature myeloid cells. Furthermore, the protease responsible for the cleavage of STAT5 in the nucleus of immature myeloid cells was not identified.

In the present study, we have purified the STAT5 protease from 32D cells and identified the protease by MS analysis. Surprisingly, the results show that the protease responsible for the conversion of STAT α to STAT γ in immature myeloid cells is CatG (cathepsin G). Unexpectedly, our results also show that the predominant form of STAT5 in 32D and FDC-P1 cells is STAT α and the truncated form is rapidly generated during the preparation of the nuclear extracts with the conventional buffers lacking CatG-specific protease inhibitors.

Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CatG, cathepsin G; CM, carboxymethyl; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, foetal bovine serum; GM-CSF, granulocyte/macrophage colony-stimulating factor; HEK-293T, human embryonic kidney-293T; hLE, human leucocyte elastase; IL, interleukin; LC/MS/MS, liquid chromatography/tandem MS; SE, size-exclusion; STAT, signal transducer and activator of transcription.

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MATERIAL AND METHODS

Cells and reagents

HEK-293T (human embryonic kidney-293T) and WEHI-3B cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Cambrex) and RPMI 1640 (PAA Laboratories) respectively, supplemented with 10% heat-inactivated FBS (foetal bovine serum; PAA Laboratories), 2 mM glutamine and 100 units/ml penicillin/streptomycin (both from Sigma-Aldrich). The cell lines 32D and FDC-P1 (a gift from Dr N. J. Dibb, Institute of Reproductive and Developmental Biology, Imperial College London, London, U.K.) were grown in RPMI 1640 supplemented with 5% WEHI-conditioned medium, 10% FBS, glutamine and penicillin/streptomycin as above. pCMV-FLAG-STAT5a has been previously described [32]. The anti-FLAG M2 antibody was purchased from Stratagene, and the anti pan-STAT5 antibody was from BD-Biosciences Pharmingen. Specific protease inhibitors, AEBSEF [4-(2-aminoethyl)benzenesulfonyl fluoride], CatG inhibitor I and elastase inhibitor IV (*N*-{2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino] benzoyl} aminoacetic acid, *N*-[*o*-(*p*-pivaloyloxybenzene) sulfonylamino benzoyl] glycine) were from Calbiochem and used at the concentrations indicated in the appropriate Figure legend. The proteases CatG and hLE (human leucocyte elastase) were from Sigma-Aldrich.

Cell transfection, preparation of cell extracts, SDS/PAGE and Western blotting

HEK-293T cells were transfected with FLAG-STAT5a using the calcium phosphate method of transfection as described previously in [33]. Total cell lysates were generated from transfected HEK-293T cells to prepare N-terminal FLAG epitope-tagged STAT5a protein for use as the substrate in cleavage assays. Following a PBS wash, cells were lysed on ice for 10 min in Nonidet P40 lysis buffer [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 50 mM sodium fluoride, 3 mM sodium orthovanadate (Sigma-Aldrich) and 1% Nonidet P40 (Calbiochem), supplemented with 1 mM DTT (dithiothreitol), 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µg/ml pepstatin (all from Roche)], frozen in liquid nitrogen, thawed at 4°C and the supernatant clarified by centrifugation (16 100 *g* for 2 min at 4°C). Cytoplasmic and nuclear extracts were prepared from 32D or FDC-P1 cells according to a standard method [30]. After a PBS wash, cells were resuspended in approximately 10 vol of hypotonic lysis buffer [10 mM Hepes (pH 7.9), 10 mM KCl and 1.5 mM MgCl₂] and incubated on ice for 10 min. Nonidet P40 was added to a final concentration of 0.1% and the sample vortexed briefly. The supernatant was collected as a cytoplasmic fraction by centrifugation (16 100 *g* for 30 min at 4°C) and the nuclear pellet resuspended in equal volumes of low-salt lysis buffer [10 mM Hepes (pH 7.9), 20 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 25% glycerol] followed by high-salt lysis buffer [20 mM Hepes (pH 7.9), 800 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 25% glycerol]. Following a 30 min incubation on ice, samples were centrifuged (16 100 *g* for 10 min at 4°C) and the supernatant removed. All of the buffers used in extract preparations contained 1 mM DTT, 1 mM sodium orthovanadate, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µg/ml pepstatin. Protein quantification was performed using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories GmbH) according to manufacturer's instructions. Alternatively cells or the nuclear fraction were lysed in boiling (95°C) 5× SDS-sample buffer [25% SDS, 25% glycerol, 12.5% 2-mercaptoethanol and a trace amount of bromophenol blue in 375 mM Tris/HCl (pH 6.8)]. Extracts were separated by SDS/PAGE, and transferred

to Immobilon PVDF membrane (Millipore). Western blots were developed using the Amersham Biosciences ECL[®] Plus chemiluminescence kit.

In vitro protease assay

HEK-293T cells were transiently transfected with FLAG-STAT5a and lysed in Nonidet P40 lysis buffer. The lysate (1 µl) was mixed with nuclear extracts from 32D cells and incubated at 37°C for 15–60 min in the presence or absence of different serine protease inhibitors. Samples were separated on SDS/PAGE and analysed by Western blotting using an anti-FLAG antibody.

Protein purification

The buffer of the nuclear extracts were exchanged to a 20 mM Tris/HCl buffer (pH 7.4) using Nap-10 columns (Amersham Biosciences) and precipitated proteins were removed by centrifugation (16 100 *g* for 10 min at 4°C). Ion-exchange was performed at room temperature (20°C) using a peristaltic pump P-1 (Amersham Biosciences). The flow rate was 2.5 ml per min. For purification, 10 mg of the proteins were fractionated on a HiPrep 16/10 DEAE FF column (Amersham Biosciences) equilibrated in 20 mM Tris/HCl buffer (pH 7.4). Proteins were eluted with a stepwise sodium chloride gradient [150–1000 mM, in 20 mM Tris buffer (pH 7.4)]. The protease activity was determined by the *in vitro* protease assay, and purification progress was analysed on SDS/PAGE (12% gel) stained with the Bio-Rad silver gel kit. The peak fraction was diluted 1:2 with 20 mM Tris buffer (pH 7.4) without sodium chloride and loaded on to a HiPrep 16/10 CM (carboxymethyl) FF column (Amersham Biosciences) equilibrated in 20 mM Tris buffer (pH 7.4). The proteins were eluted and analysed as described above. The fraction with peak activity was concentrated 15-fold in Amicon Ultra centrifuge tubes (Millipore). The concentrated proteins were separated on a 16% silver gel, and 1 ml was applied to a HiLoad 16/60 Superdex 75 prep grade SE (size-exclusion) column (Amersham Biosciences) equilibrated in 20 mM Tris buffer (pH 7.4) containing 150 mM sodium chloride. The SE column was calibrated with the Low Molecular Weight Gel Filtration Calibration Kit from Amersham Biosciences including ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa) and Blue Dextran 2000. The SE column run was performed with the Biologic Duo Flow FPLC system from Bio-Rad at 4°C and a flow rate of 1 ml per min.

LC/MS/MS (liquid chromatography/tandem MS) identification

The corresponding bands from two gels were pooled together before enzymatic digestion thereby increasing the total amount of protein present for enhanced detection by MS. In-gel reduction, alkylation and digestion with trypsin were performed prior to subsequent analysis by MS. Cysteine residues were reduced with DTT and derivatized by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion was carried out overnight at room temperature after an initial 2 h incubation at 37°C.

For LC/MS/MS, peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and freeze-dried. Each sample was then resuspended in 23 µl of 50 mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex). Peptides were resolved by reversed phase chromatography on a 75 µm C18 PepMap column. A gradient of acetonitrile in 0.05% formic

acid was delivered to elute the peptides at a flow rate of 200 nl/min. Peptides were ionized by electrospray ionization using a Z-spray source fitted to a QToF-micro (Waters Corporation). The instrument was set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation. The MS/MS (tandem MS) analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide.

The mass spectral data was processed into peptide peak lists and searched against the Swiss-Prot or NCBI non-redundant databases using Mascot software (Matrix Science). The data was searched using specific amino acid modification parameters, ie. variable cysteine carbamidomethylation modification (resulting from reduction and alkylation reaction), variable methionine oxidation modification, phosphorylation (of serine, threonine and tyrosine residues), ubiquitination, N-acetylation and glycosylation (raw MS data was checked for the presence of glycan modification). The data was also searched using a non-specific enzyme parameter. This parameter enables the identification of non-tryptic peptides, which may be present if there is for example N- or C-terminal cleavage of proteins. MS/MS sequence information was obtained for all the peptides included in the results.

RESULTS

Purification of the STAT5 protease

In the present study we wished to understand more clearly the molecular regulation of STAT5 by proteolytic cleavage and its importance in different cell types by identifying the protease(s) responsible for the conversion of STAT5 α to STAT5 γ . Previously, it has been shown that immature myeloid cell lines such as FDC-P1 and 32D contain nuclear STAT5 γ and, therefore we used the 32D cell line to identify the STAT5 protease in these cells. We initially confirmed previous findings that 32D cells contain nuclear STAT5 γ by lysing cells in boiling SDS-sample buffer or Nonidet P40 lysis buffer, followed by Western blot analysis of lysates using a pan-STAT5 antibody. When cells were lysed in boiling SDS-sample buffer, the majority of STAT5 detected was STAT5 α , and only a small amount of STAT5 γ was detected (Figure 1A, lanes 1–3). In contrast, when the lysates were prepared in Nonidet P40 lysis buffer, most of the STAT5 proteins were STAT5 γ (Figure 1A, lanes 4–6). This confirmed previous findings that immature myeloid cells contain truncated STAT5 proteins, albeit at a considerably lower amount than STAT5 α [20]. Using an *in vitro* protease assay, with FLAG-tagged STAT5 α as a substrate, we detected the STAT5 protease activity in the nuclear, but not cytoplasmic, extracts of 32D cells (Figure 1B, lanes 1 and 2). To confirm that this proteolytic activity belonged to the serine protease family, as previously described, we performed the *in vitro* protease assay in the presence or absence of AEBSF (Figure 1C). The STAT5 protease activity was inhibited by 4 mM AEBSF in the *in vitro* protease cleavage assay (Figure 1C, lanes 2, 4, 6 and 8). Furthermore, STAT5 α cleavage activity was clearly detected using low amounts of cell extracts (5–0.625 μ g; Figure 1C, lanes 1, 3, 5 and 7), and also in the presence of 4 mM AEBSF (lanes 2 and 4). These results suggest that the proteolytic activity is abundantly present or highly active in these immature myeloid cells and requires higher concentrations of AEBSF to fully inhibit the activity of the protease.

Based on the results depicted in Figure 1(B), we next used only the nuclear extracts for purification of the protease via ion-exchange on a DEAE column followed by a CM column. Utilizing the *in vitro* assay to evaluate the STAT5-specific protease activity, maximal cleavage activity was recovered in the fraction

Figure 1 Truncated STAT5 is generated by a protease which is found in nuclear extracts

(A) 32D cells were lysed in equal volumes of boiling SDS-sample buffer (lanes 1–3) or conventional Nonidet P40 (NP-40) lysis buffer (lanes 4–6). Proteins from 10, 5 and 1 μ l of the lysates were separated on SDS/PAGE and the STAT5 proteins were analysed by immunoblotting using an anti-pan-STAT5 antibody, detecting full-length and C-terminal-truncated STAT5. (B) Cellular (25 μ g) or nuclear (5 μ g) extracts (lanes 1 and 2 respectively) from 32D cells were mixed with FLAG-STAT5 α (HEK-293T cell lysate, 5 μ g) in a total volume of 40 μ l and incubated for 15 min at 37°C. Then, 20 μ l were separated on SDS/PAGE followed by immunoblotting with an anti-FLAG antibody. As a control, FLAG-STAT5 α -containing lysate was incubated for 15 min at 37°C alone (lane 3). CE, cellular extract; NE, nuclear extract. (C) Various amounts of 32D cell nuclear extracts were incubated with FLAG-STAT5 α (HEK-293T cell lysate, 5 μ g) in a total volume of 20 μ l for 20 min at 37°C in the presence or absence of 4 mM AEBSF before separating by SDS/PAGE followed by immunoblotting with an anti-FLAG antibody. As a control, FLAG-STAT5 α -containing lysates were incubated for 20 min at 37°C alone (lane 9). WB, Western blot.

eluted with 150 mM sodium chloride from the DEAE column (Figure 2A, lane 4). The semi-purified protease activity was at high enough levels in this fraction so that the truncated FLAG-STAT5 product was not observed under the experimental conditions used in the *in vitro* protease assay (1 h incubation at 37°C), most likely due to removal of the FLAG-tag by over-digestion at additional secondary sites. The 150 mM fraction was next loaded and separated on the second CM column. The STAT5 protease was eluted with 350 and 450 mM sodium chloride, with peak activity detected in the fraction eluted with 450 mM sodium chloride (Figure 2B, lane 7). The silver stained gel of the eluted fractions, including the active fractions at 350 and 450 mM NaCl, did not clearly reveal specific proteins (Figure 2B, lanes 4–11). However, following concentration of the 450 mM fraction 15-fold,

Figure 2 Purification of the STAT5 protease

To assess the progress of the STAT5 protease activity during purification, 23 μ l of each fraction from the ion-exchange columns were separated by SDS/PAGE (12% gel) and the proteins visualized by silver staining. STAT5 protease activity was analysed using the *in vitro* cleavage assay. Therefore 23 μ l of each fraction were mixed with 1 μ l of HEK-293T cell lysates containing FLAG-STAT5a and incubated for 60 min at 37°C, followed by immunoblotting with an anti-FLAG antibody. **(A)** Proteins separated by the DEAE-column, silver stained (lanes 1–9, upper panel) and protease cleavage assay (lower panel). Peak activity of the STAT5 protease was obtained in the fraction eluted with 150 mM NaCl (lane 4). **(B)** Proteins from the fraction eluted with 150 mM NaCl from the DEAE-column separated on the CM column. Silver staining is shown in the upper panel and the result of the protease cleavage assay in the lower panel (lanes 1–11). The fraction eluted with 450 mM NaCl (lane 7) from the CM column was concentrated 15-fold and 23 μ l was separated by SDS/PAGE (16% gel; lane 12). The two bands visible after silver staining are indicated with arrows. **(C)** Murine CatG (P28293; EC 3.4.21.20, vimentin-specific protease) was identified as a single protein in both bands analysed by MS. The sequence coverage was 56% and matched peptides are shown in bold. WB, Western blot; F/t, flow-through.

two bands were visible between the 22 kDa and 36 kDa protein marker bands in a SDS-gel after silver staining (Figure 2B, lane 12). The exact size of these bands was determined by SE chromatography, which yielded a single peak with a light shoulder and a calculated molecular mass of about 23.5 kDa and 25.5 kDa (results not shown). This is consistent with the results from a previous purification of STAT5 protease from FDC-P1 cells [22]. Because it was not clear which of the two bands corresponded to the STAT5 protease, an aliquot of the concentrated sample was mixed with the serine protease inhibitor AEBSF coupled to beads, precipitated and analysed on a silver gel. Surprisingly, both bands

were found in the precipitate (results not shown), indicating that they both contained active serine proteases.

Identification of the purified proteins by LC/MS/MS

Analysis of the two bands revealed the presence of a single identical protein in each band. The previously unknown STAT5 protease was identified as murine CatG precursor (P28293; EC 3.4.21.20, vimentin-specific protease) with 19 peptides matching and with sequence coverage of 56% (Figure 2C). Searching the data with the non-specific enzyme parameter did not yield any further increase in sequence coverage. Additionally, no evidence for ubiquitination, phosphorylation, N-acetylation or glycosylation was found in these experiments (results not shown). From the different parameters used to search the data, there is no obvious explanation as to why the protein resolved as a doublet.

CatG is responsible for the proteolytic conversion of STAT5

We incubated N-terminal tagged FLAG-STAT5a with nuclear extracts or various amounts of purified CatG to verify that CatG was the protease that cleaved STAT5 in 32D cells. The resulting STAT5 isoforms were analysed by immunoblotting with an anti-FLAG antibody (Figure 3A). As shown in Figure 3(A), STAT5 γ generated by purified CatG is of the same size as STAT5 cleaved by the protease in 32D nuclear extracts (Figure 3A, lane 1 compared with lanes 2–4). In contrast, the cleavage pattern of STAT5 generated by purified hLE appears similar, but the truncated form observed was subtly smaller (Figure 3A, lane 1 compared with lanes 5–7). To exclude the possibility that hLE is also involved in the proteolytic processing of STAT5 in 32D cells, we used specific inhibitors to block the activity of these two proteases (Figure 3B). The protease inhibitor specific for CatG blocked the activity of CatG to cleave STAT5a in the *in vitro* protease assay (Figure 3B, lanes 1 and 2), whereas the specific hLE inhibitor did not block this activity (Figure 3B, lanes 1 and 3).

Using 32D nuclear extract instead of purified CatG or hLE, the protease activity cleaving STAT5 was strongly inhibited by 2.5 and 25 μ M CatG inhibitor and completely blocked with 250 μ M CatG inhibitor (Figure 3C, lanes 2–4). In contrast, there was no reduced protease activity in the presence of 250 μ M hLE-specific inhibitor (Figure 3C, lanes 6 and 7), whereas even 25 μ M hLE inhibitor could inhibit the activity of purified hLE (Figure 3C, lanes 8 and 9). Together, these results demonstrate that CatG is the specific protease that generates STAT5 γ in 32D cell lysates.

Previously, we observed that incubation of N-terminal tagged FLAG-STAT5a with 32D nuclear extracts containing higher amounts of the STAT5a-protease in the *in vitro* cleavage assay resulted in undetectable levels of FLAG-STAT5a (Figure 2A, lane 4). We hypothesized that this was due to over-digestion of FLAG-STAT5 at higher protease concentrations. To confirm this, we repeated the *in vitro* cleavage assay under the same conditions as before (1 h at 37°C), but with various amounts of purified CatG instead of 32D cell nuclear extract. As shown in Figure 3(D), the amount of FLAG-STAT5a (α or γ) detected was strongly reduced with increasing amounts of CatG added (Figure 3D, lanes 2–4) and was completely undetectable in the presence of 10 m-units of CatG (Figure 3D, lane 1). This indicated that CatG can cleave at additional cleavage sites in the STAT5a protein in a dose-dependent manner.

STAT5 is full-length in the nucleus of immature myeloid cells and STAT5 γ is generated upon lysis of cells

CatG is normally stored in azurophilic granules in the cytoplasm. With the surprising identification of CatG as the protease

Figure 3 Comparison of the STAT5 cleavage pattern by the protease in 32D cell nuclear extracts with that of CatG or hLE via the *in vitro* cleavage assay

(A) Nuclear extract (2.5 μ g; lane 1) or various amounts of CatG (lanes 2–4) or hLE (lanes 5–7) were mixed with 1 μ l of HEK-293T cell lysate containing FLAG-STAT5a in a total volume of 20 μ l and incubated for 20 min at 37 $^{\circ}$ C, followed by immunoblotting with an anti-FLAG antibody. As a control, the HEK-293T cell lysate was incubated for 20 min at 37 $^{\circ}$ C alone (lane 8). (B) The STAT5 protease was inhibited by a protease inhibitor specific for CatG but not by an hLE inhibitor in the *in vitro* cleavage assay. CatG (2.5 m-units) was mixed with 1 μ l of HEK-293T cell lysate containing FLAG-STAT5a alone (lane 1) or in the presence of 25 μ M of the CatG inhibitor (lane 2) or 25 μ M of the hLE inhibitor (lane 3) and incubated for 30 min at 37 $^{\circ}$ C, followed by immunoblotting with an anti-FLAG antibody. As a control, the HEK-293T cell lysate was incubated for 30 min at 37 $^{\circ}$ C alone (lane 4). (C) 32D cell nuclear extract (2.5 μ g) was mixed with 1 μ l of HEK-293T cell lysate containing FLAG-STAT5a alone (lanes 1, 6 and 8) or in the presence of the CatG inhibitor (2.5–250 μ M; lanes 2–4) or 250 μ M of the hLE inhibitor (lane 7) and incubated for 30 min at 37 $^{\circ}$ C, followed by immunoblotting with an anti-FLAG antibody. As a control, the HEK-293T cell lysate was incubated for 30 min at 37 $^{\circ}$ C alone (lanes 5 and 10). The protease activity in the 32D nuclear extract was blocked in the presence of 250 μ M of the CatG inhibitor, whereas the addition of an hLE inhibitor showed no effect. (D) Various amounts of CatG (lanes 1–4) were mixed with 1 μ l of HEK-293T cell lysate containing FLAG-STAT5a in a total volume of 20 μ l and incubated for 60 min at 37 $^{\circ}$ C, followed by immunoblotting with an anti-FLAG antibody. As a control, the HEK-293T cell lysate was incubated for 60 min at 37 $^{\circ}$ C alone (lane 5). WB, Western blot; NE, nuclear extract.

responsible for the conversion of STAT5 in 32D cell nuclear extracts, we wanted to clarify whether the detection of STAT5 γ , rather than STAT5 α in this compartment was due to an authentic *in vivo* biological processing event or instead due to artificial exposure of STAT5 to highly active CatG during cell lysate preparation. Therefore we prepared cytosolic and nuclear extracts by standard methods and also in the presence of 4 mM AEBSF or the specific CatG inhibitor in all of the buffers. In contrast with AEBSF (which inhibits multiple serine proteases) the CatG inhibitor allowed us to specifically block CatG activity. We also included one sample of nuclear extract prepared in boiling SDS-sample buffer, in which all of the proteases are denatured. As shown in Figure 4, in both 32D and FDC-P1 cells, when the extracts were prepared in the presence of the specific CatG inhibitor, only STAT5 α was detected by Western blotting for STAT5 (Figure 4, lanes 3 and 8). In contrast, the nuclear extracts prepared without inhibitors (Figure 4, lanes 1 and 5) or with the serine protease inhibitor AEBSF alone (Figure 4, lanes 2 and 6) contained predominantly STAT5 γ . Furthermore, the addition of AEBSF and the CatG-specific inhibitor together did not increase the amount of STAT5 α detected over the addition of the CatG-specific inhibitor alone (Figure 4, lanes 7 and 8). The finding that AEBSF did not fully inhibit the proteolytic cleavage of STAT5 by CatG in these cells was not surprising given the results in

Figure 4 Comparison of STAT5 present in nuclear extracts using different conditions during the preparation of extracts

Nuclear extracts (20 μ l) prepared from 32D (left panel) or FDC-P1 (right panel) cells were separated by SDS/PAGE followed by Western blotting using an anti-pan-STAT5 antibody. Extracts were prepared using the standard method (lanes 1 and 5), with 4 mM AEBSF (lanes 2 and 6), with 4 mM AEBSF and 250 μ M CatG inhibitor (lane 7) or with 250 μ M CatG inhibitor alone (lanes 3 and 8) in all buffers. Additionally the extracts were prepared with the standard method, but the nuclei were lysed in boiling SDS-sample buffer (lanes 4 and 9). The full-length and truncated forms of STAT5 are indicated with arrows. WB, Western blot; NE, nuclear extract.

Figure 1(C), which suggests that very high concentrations of this general serine protease inhibitor would be required to fully inactivate all active serine proteases. As before, in the samples prepared with boiling SDS-sample buffer (Figure 4, lanes 4 and

9), the amount of STAT5 γ detected was markedly reduced and the full-length form increased. Thus nuclear STAT5 γ is specifically generated by the action of CatG during cell lysate preparation and does not occur *in vivo* in intact immature myeloid cells.

DISCUSSION

STAT5 is the signal transducer for the IL-3 and IL-2 family of cytokines. In contrast with mature myeloid cells, in several immature myeloid lineages STAT5 γ isoforms lacking the C-terminus were found to be predominantly expressed rather than the full-length form [18–20,22,31]. The C-terminus of STAT5 contains the transactivation domain and plays a role in transcriptional activation and, consistent with the detection of STAT5 γ , immature myeloid cells express only a subset of genes activated by STAT5 α in mature cells [20]. Moreover, in acute myeloid leukaemia patients a change in constitutive STAT3 and STAT5 expression from full-length to a C-terminally truncated form was significantly associated with disease progression and relapse [34]. Therefore unravelling the underlying mechanism that mediates STAT processing enhances our understanding of the regulation of STAT proteins in normal haematopoiesis and its potential dysregulation in abnormal malignant haematopoiesis. Therefore in the present study we decided to investigate this regulation in normal myeloid cell differentiation.

Previously, it has been shown that STAT5 γ is generated from the full-length form by limited proteolysis [18,20,22,23,34]. The STAT5 protease responsible was constitutively active in the nucleus of immature myeloid cells and cleaved independently of the activation state of STAT5. This unusual nuclear STAT5 specific protease was characterized and shown to be sensitive to PMSF, a broad-spectrum serine protease inhibitor, but not to aprotinin, pepstatin, benzamide [22] or ONO-5046, a specific inhibitor of hLE and STAT6 protease in mast cells [30]. The size of the STAT5 protease was predicted to be 25 kDa, and activity was observed in a wide range of physiological pH values. The cleavage site in murine STAT5 was mapped between Tyr⁷²⁰ and Met⁷²¹ in STAT5a and Tyr⁷²⁴ and Met⁷²⁵ in STAT5b, and mutation of these residues perturbed STAT5 function in myeloid cell maturation [22,31]. We have purified the STAT5 protease from nuclear extracts prepared from the immature myeloid cell line 32D and identify it as CatG, which is expressed during the promyeloid stage of neutrophil differentiation [35]. The characteristics of CatG [36] match perfectly with the characteristics described for the STAT5 protease in size, sensitivity and specificity. There are several possible cleavage sites for CatG in the STAT5 protein and, consistent with this our results show a second band for STAT5 γ , depending on the concentration of the protease in the extracts and the incubation time. Indeed, in some cases, N-terminal-tagged STAT5 was not detected by the FLAG-antibody, indicating at least one more cleavage site near the N-terminus used by CatG (results not shown).

Together with leucocyte elastase, proteinase 3 and azurocidine, a proteolytically inactive protease homologue, CatG belongs to a superfamily of azurophilic granule serine proteases. These proteases are expressed as inactive proforms (zymogens) which are activated by dipeptidylpeptidase I [37], and the active enzymes are stored in azurophilic granules of haematopoietic cells [38,39]. It is possible that the two bands we obtained after purification represent active processed forms of CatG with a size of about 23.5 kDa and 25.5 kDa, because both bands were precipitated with the serine protease inhibitor AEBSF coupled to beads, while CatG was the only protein identified by MS. To exclude the possibility that other serine proteases from the azurophilic granule

were responsible for the conversion of STAT5 α we used purified hLE and specific inhibitors for CatG and hLE. The hLE is able to process STAT5 with a similar cleavage pattern to that of CatG, but with the specific inhibitors we could clearly show that only CatG is responsible for the conversion of STAT5 in 32D cell nuclear extracts.

It is most likely that CatG, which is stored in cytoplasmic granules, is pelleted together with the nuclei during the standard method for preparing cytoplasmic and nuclear extracts. In this case, CatG is only a contaminant of the nuclear fraction as it was clearly shown for hLE in neutrophils cleaving STAT3 [27]. However, CatG has also been found in the nucleus of cells; in the leukaemic cell line NB4, Biggs et al. [40] demonstrated that the human brm (brahma) protein is cleaved by CatG during apoptosis when CatG was released from the granule of NB4 cells and became diffusely distributed. Because CatG is found in the nucleus only after induction of apoptosis, whereas the truncated form of STAT5 is thought to be generated in the nucleus of uninduced cells, we wanted to determine whether the proteolytic event occurs in the living cell or during the preparation of the cytoplasmic and nuclear extracts. Our results clearly show that full-length STAT5 is cleaved during the preparation of the extracts without inhibitors for CatG. With the conventional buffers, only STAT5 γ was detected by Western blotting, and the addition of AEBSF did not yield STAT5 α . This alone would suggest that STAT5 γ is generated in the nucleus of intact immature myeloid cells, because the presence of 4 mM AEBSF should block all serine protease activity in the extracts, as shown in Figure 1(C). But also in this *in vitro* cleavage assay, where the nuclear extract was 20 $\times$ diluted in the sample mix (Figure 1C, lanes 1 and 2), the CatG activity was not completely blocked. It is possible that the concentration of 4 mM AEBSF, used in various studies, was not high enough to block the activity of all the serine proteases (including CatG) in these cells during lysis. In contrast, the STAT5 cleavage was completely blocked by the specific CatG inhibitor. This strongly indicates that the limited proteolytic conversion of STAT5 α to C-terminally truncated STAT5 is mediated by CatG and occurs during the preparation of the extracts from 32D and FDC-P1 cells. Similar results were shown for the conversion of STAT3 by hLE in neutrophils [27]. When the nuclear fraction was lysed in boiling SDS-sample buffer inconsistent results were obtained; in all cases STAT5 α was significantly increased, but in some cases STAT5 γ was also present. While this is a standard method to lyse cells and inactivate proteases simultaneously, the results may be misleading when the cells contain high amounts of proteases. The identification of the STAT5 protease allowed us to use a specific inhibitor to selectively and completely block CatG activity during the preparation of the extracts, yielding more conclusive results than lysis with boiling SDS-sample buffer. From our results we cannot exclude the possibility that under certain normal (such as apoptosis) or disease situations, leakage of active CatG into the nucleus may cleave STAT5, but we can exclude this for IL-3-stimulated 32D and FDC-P1 cells. Thus our results strongly suggest that full-length STAT5 rather than truncated STAT5 is essential for myeloid differentiation.

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REFERENCES

- 1 Levy, D. E. and Darnell, Jr, J. E. (2002) Stats: transcriptional control and biological impact. *Nat. Rev. Mol. Cell. Biol.* **3**, 651–662

- 2 Leonard, W. J. and O'Shea, J. J. (1998) Jaks and STATs: biological implications. *Annu. Rev. Immunol.* **16**, 293–322
- 3 Darnell, Jr, J. E. (1997) STATs and gene regulation. *Science* **277**, 1630–1635
- 4 Ihle, J. N. (1995) Cytokine receptor signalling. *Nature* **377**, 591–594
- 5 Mui, A. L., Wakao, H., Harada, N., O'Farrell, A. M. and Miyajima, A. (1995) Interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 transduce signals through two forms of STAT5. *J. Leukoc. Biol.* **57**, 799–803
- 6 Mui, A. L., Wakao, H., O'Farrell, A. M., Harada, N. and Miyajima, A. (1995) Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J.* **14**, 1166–1175
- 7 Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* **11**, 179–186
- 8 Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J. and Davey, H. W. (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7239–7244
- 9 Imada, K., Bloom, E. T., Nakajima, H., Horvath-Arcidiacono, J. A., Udy, G. B., Davey, H. W. and Leonard, W. J. (1998) Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J. Exp. Med.* **188**, 2067–2074
- 10 Feldman, G. M., Rosenthal, L. A., Liu, X., Hayes, M. P., Wynshaw-Boris, A., Leonard, W. J., Hennighausen, L. and Finbloom, D. S. (1997) STAT5a-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. *Blood* **90**, 1768–1776
- 11 Nakajima, H., Liu, X. W., Wynshaw-Boris, A., Rosenthal, L. A., Imada, K., Finbloom, D. S., Hennighausen, L. and Leonard, W. J. (1997) An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor α -chain induction. *Immunity* **7**, 691–701
- 12 Hoelbl, A., Kovacic, B., Kerenyi, M. A., Simma, O., Warsch, W., Cui, Y., Beug, H., Hennighausen, L., Moriggl, R. and Sexl, V. (2006) Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. *Blood* **107**, 4898–4906
- 13 Snow, J. W., Abraham, N., Ma, M. C., Abbey, N. W., Herndier, B. and Goldsmith, M. A. (2002) STAT5 promotes multilineage hematolymphoid development *in vivo* through effects on early hematopoietic progenitor cells. *Blood* **99**, 95–101
- 14 Yao, Z., Cui, Y., Watford, W. T., Bream, J. H., Yamaoka, K., Hissong, B. D., Li, D., Durum, S. K., Jiang, Q., Bhandoola, A. et al. (2006) Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 1000–1005
- 15 Ilaria, Jr, R. L., Hawley, R. G. and Van Etten, R. A. (1999) Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation. *Blood* **93**, 4154–4166
- 16 Bovolenta, C., Testolin, L., Benussi, L., Lievens, P. M. and Liboi, E. (1998) Positive selection of apoptosis-resistant cells correlates with activation of dominant-negative STAT5. *J. Biol. Chem.* **273**, 20779–20784
- 17 Lokuta, M. A., McDowell, M. A. and Paulnock, D. M. (1998) Identification of an additional isoform of STAT5 expressed in immature macrophages. *J. Immunol.* **161**, 1594–1597
- 18 Meyer, J., Jucker, M., Ostertag, W. and Stocking, C. (1998) Carboxyl-truncated STAT5 β is generated by a nucleus-associated serine protease in early hematopoietic progenitors. *Blood* **91**, 1901–1908
- 19 Azam, M., Erdjument-Bromage, H., Kreider, B. L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J. N. and Schindler, C. (1995) Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J.* **14**, 1402–1411
- 20 Azam, M., Lee, C., Strehlow, I. and Schindler, C. (1997) Functionally distinct isoforms of STAT5 are generated by protein processing. *Immunity* **6**, 691–701
- 21 Rothman, P., Kreider, B., Azam, M., Levy, D., Wegenka, U., Eilers, A., Decker, T., Horn, F., Kashleva, H., Ihle, J. et al. (1994) Cytokines and growth factors signal through tyrosine phosphorylation of a family of related transcription factors. *Immunity* **1**, 457–468
- 22 Lee, C., Piazza, F., Brutsaert, S., Valens, J., Strehlow, I., Jarosinski, M., Saris, C. and Schindler, C. (1999) Characterization of the Stat5 protease. *J. Biol. Chem.* **274**, 26767–26775
- 23 Xia, Z., Salzler, R. R., Kunz, D. P., Baer, M. R., Kazim, L., Baumann, H. and Wetzler, M. (2001) A novel serine-dependent proteolytic activity is responsible for truncated signal transducer and activator of transcription proteins in acute myeloid leukemia blasts. *Cancer Res.* **61**, 1747–1753
- 24 Caldenhoven, E., van Dijk, T. B., Raaijmakers, J. A., Lammers, J. W., Koenderman, L. and de Groot, R. P. (1999) Activation of a functionally distinct 80-kDa STAT5 isoform by IL-5 and GM-CSF in human eosinophils and neutrophils. *Mol. Cell. Biol. Res. Commun.* **1**, 95–101
- 25 Chakraborty, A. and Tweardy, D. J. (1998) Granulocyte colony-stimulating factor activates a 72-kDa isoform of STAT3 in human neutrophils. *J. Leukoc. Biol.* **64**, 675–680
- 26 Epling-Burnette, P. K., Garcia, R., Bai, F., Ismail, S., Loughran, Jr, T. P., Djeu, J. Y., Jove, R. and Wei, S. (2002) Carboxy-terminal truncated STAT5 is induced by interleukin-2 and GM-CSF in human neutrophils. *Cell. Immunol.* **217**, 1–11
- 27 Kato, T., Sakamoto, E., Kutsuna, H., Kimura-Eto, A., Hato, F. and Kitagawa, S. (2004) Proteolytic conversion of STAT3 α to STAT3 γ in human neutrophils: role of granule-derived serine proteases. *J. Biol. Chem.* **279**, 31076–31080
- 28 Sherman, M. A., Powell, D. R. and Brown, M. A. (2002) IL-4 induces the proteolytic processing of mast cell STAT6. *J. Immunol.* **169**, 3811–3818
- 29 Sherman, M. A., Secor, V. H. and Brown, M. A. (1999) IL-4 preferentially activates a novel STAT6 isoform in mast cells. *J. Immunol.* **162**, 2703–2708
- 30 Suzuki, K., Nakajima, H., Kagami, S., Suto, A., Ikeda, K., Hirose, K., Hiwasa, T., Takeda, K., Saito, Y., Akira, S. and Iwamoto, I. (2002) Proteolytic processing of Stat6 signaling in mast cells as a negative regulatory mechanism. *J. Exp. Med.* **196**, 27–38
- 31 Piazza, F., Valens, J., Lagasse, E. and Schindler, C. (2000) Myeloid differentiation of FdCP1 cells is dependent on Stat5 processing. *Blood* **96**, 1358–1365
- 32 John, S., Vinkemeier, U., Soldaini, E., Darnell, Jr, J. E. and Leonard, W. J. (1999) The significance of tetramerization in promoter recruitment by Stat5. *Mol. Cell Biol.* **19**, 1910–1918
- 33 Meyer, T., Hendry, L., Begitt, A., John, S. and Vinkemeier, U. (2004) A single residue modulates tyrosine dephosphorylation, oligomerization, and nuclear accumulation of stat transcription factors. *J. Biol. Chem.* **279**, 18998–19007
- 34 Xia, Z., Sait, S. N., Baer, M. R., Barcos, M., Donohue, K. A., Lawrence, D., Ford, L. A., Block, A. M., Baumann, H. and Wetzler, M. (2001) Truncated STAT proteins are prevalent at relapse of acute myeloid leukemia. *Leuk. Res.* **25**, 473–482
- 35 Hanson, R. D., Connolly, N. L., Burnett, D., Campbell, E. J., Senior, R. M. and Ley, T. J. (1990) Developmental regulation of the human cathepsin G gene in myelomonocytic cells. *J. Biol. Chem.* **265**, 1524–1530
- 36 Wiedow, O. and Meyer-Hoffert, U. (2005) Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. *J. Intern. Med.* **257**, 319–328
- 37 McGuire, M. J., Lipsky, P. E. and Thiele, D. L. (1993) Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. *J. Biol. Chem.* **268**, 2458–2467
- 38 Gullberg, U., Andersson, E., Garwicz, D., Lindmark, A. and Olsson, I. (1997) Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development. *Eur. J. Haematol.* **58**, 137–153
- 39 Levy, O. (1996) Antibiotic proteins of polymorphonuclear leukocytes. *Eur. J. Haematol.* **56**, 263–277
- 40 Biggs, J. R., Yang, J., Gullberg, U., Muchardt, C., Yaniv, M. and Kraft, A. S. (2001) The human brm protein is cleaved during apoptosis: the role of cathepsin G. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3814–3819

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