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PURIFICATION AND IDENTIFICATION OF THE STAT5 PROTEASE IN MYELOID CELLS

Running title: Generation of truncated STAT5

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ABSTRACT

Signal transducer and activator of transcription (STAT) 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 was shown that STAT5 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 mass spectrometric analysis as the granule-derived serine protease, Cathepsin G (CatG). We show that purified CatG can specifically cleave full-length STAT5 to generate STAT5 γ , and this activity can be inhibited by AEBSF 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 only STAT5 α . These studies indicate that nuclear STAT5 γ does not naturally exist in immature myeloid cells and is artificially generated from STAT5 α during the preparation of extracts due to the abundance of CatG in these cells. Therefore, in contrast to earlier studies, our data suggest that STAT5 α , rather than STAT5 γ is the active form in immature myeloid cells.

INTRODUCTION

Signal transducer and activator of transcription (STAT) 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, granulocyte-macrophage colony-stimulating factor (GM-CSF) and thrombopoietin or 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 granulocyte-colony-stimulating factor (G-CSF)-induced differentiation of 32D cells [15]. In myeloid progenitors, IL-3 stimulates the activation of C-terminally truncated isoforms of STAT5a (77 kD) and STAT5b (80 kD), collectively called STAT5 γ [16-21]. In immature myeloid cells the STAT5 γ isoforms are predominantly localized to the nucleus and activates only a subset of genes activated by STAT5 α in mature myeloid cells [18, 20, 22]. It was demonstrated that STAT5 γ is generated by limited proteolytic processing of STAT5 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 phenylmethylsulfonyl fluoride (PMSF). Similar protease activities with the capacity to cleave STAT3 and STAT5 were also 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 STAT5 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 this study, we purified the STAT5 protease from 32D cells and identified the protease by mass spectrometry analysis. Surprisingly, the results show that the protease responsible for the conversion of STAT5 α to STAT5 γ in immature myeloid cells is CatG. Unexpectedly, our results also show that the predominant form of STAT5 in 32D and FDC-P1 cells is STAT5 α and the truncated form is rapidly generated during the preparation of the nuclear extracts with the conventional buffers lacking CatG specific protease inhibitors.

MATERIAL AND METHODS

Cells and Reagents

293T and WEHI-3B cells were maintained in DMEM (Cambrex, Verviers, Belgium) and RPMI 1640 (PAA Laboratories, Pasching, Austria) respectively, supplemented with 10 % heat inactivated fetal bovine serum (PAA Laboratories), 2 mM glutamine and 100 units/ml penicillin-streptomycin (both from Sigma-Aldrich, Dorset, UK). The cell lines 32D and FDC-P1 (a

generous gift from Dr. N. J. Dibb) were grown in RPMI 1640 supplemented with 5 % WEHI conditioned medium, 10 % FBS (PAA Laboratories), glutamine and penicillin-streptomycin (both from Sigma-Aldrich) as before. pCMV-FLAG-STAT5a has been previously described [32]. The anti-FLAG M2 antibody was purchased from Stratagene (Amsterdam, The Netherlands), and the anti pan-Stat5 antibody was from BD-Biosciences Pharmingen (San Diego, CA, USA). Specific protease inhibitors, AEBSF, cathepsin G Inhibitor I and Elastase Inhibitor IV (N-(2-(4-(2,2-Dimethylpropionyloxy) phenylsulfonylamino) benzoyl) aminoacetic acid N-(o-(p-Pivaloyloxybenzene) sulfonylamino) benzoyl) glycine) were all from Calbiochem (Darmstadt, Germany) and used at the concentrations indicated in the appropriate figure. The proteases Cathepsin G and human Leukocyte Elastase were from Sigma-Aldrich.

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

293T cells were transfected with FLAG-STAT5a by the calcium phosphate method of transfection as described previously [33]. Total cell lysates were generated from transfected 293T cells to prepare N-terminal FLAG epitope-tagged STAT5a protein for use as substrate in cleavage assays. Following a PBS wash, cells were lysed on ice for 10 min in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM sodium fluoride, 3 mM sodium orthovanadate (Sigma-Aldrich) and 1 % NP-40 (Calbiochem), supplemented with 1 mM DTT, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µg/ml pepstatin (all from Roche, East Sussex, UK) frozen in liquid nitrogen, thawed at 4°C and the supernatant clarified by centrifugation. 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 volumes 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. NP-40 was added to a final concentration of 0.1% and the sample vortexed briefly. The supernatant was collected as a cytoplasmic fraction by centrifugation 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 minute incubation on ice, samples were centrifuged and the supernatant taken. All of the buffers used in extract preparations contained 1 mM DTT, 1 mM sodium orthovanadate (Sigma-Aldrich), 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µg/ml pepstatin (all from Roche). Protein quantification was performed according to manufacturer's instructions, using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories GmbH, Muenchen, Germany). Alternatively cells or the nuclear fraction were lysed in boiling (95°C) 5x SDS-sample buffer (25% SDS, 25% glycerol,

12.5% 2-mercaptoethanol, and a trace amount of bromphenol blue in 375 mM Tris-HCl, pH 6.8) Extracts were separated by SDS-PAGE, and transferred to Immobilon PVDF membrane (Millipore, Bedford, USA). Western blots were developed using the Amersham Biosciences ECL Plus chemiluminescence kit.

***In-vitro* protease assay**

293T cells were transiently transfected with FLAG-STAT5a and lysed in NP-40 lysis buffer. 1 μ l of the lysate was mixed with nuclear extracts from 32D cells and incubated at 37°C for 15- 60 minutes in the presence or absence of different serine protease inhibitors. Samples were separated on SDS-PAGE and analyzed by Western blotting using an anti-FLAG antibody.

Protein Purification

The buffer of the nuclear extracts were exchanged to a 20 mM Tris buffer, pH 7.4 using Nap-10 columns (Amersham Biosciences, Buckinghamshire, UK) and precipitated proteins were removed by centrifugation. Ion exchange was performed at room temperature using the peristaltic pump P-1 (Amersham Biosciences). The flow rate was 2.5 ml per minute. For purification, 10 mg of the proteins were fractionated on a HiPrep 16/10 DEAE FF column (Amersham Biosciences) equilibrated in 20 mM Tris 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 a 12 % SDS-gel stained with the Biorad silver gel kit. The peak fraction was diluted 1:2 with 20 mM Tris, pH 7.4 without sodium chloride and loaded to a HiPrep 16/10 CM FF column (Amersham Biosciences) equilibrated in 20 mM Tris, pH 7.4. The proteins were eluted and analysed as described above. The fraction with peak activity was concentrated 15 fold by Amicon Ultra centrifuge tubes (Millipore). The concentrated proteins were separated in a 16 % silver gel, and 1 ml was applied to a HiLoad 16/60 Superdex 75 prep grade size exclusion (SE) column (Amersham Biosciences) equilibrated in 20 mM Tris, pH 7.4 buffer 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 kD), Chymotrypsinogen A (25 kD), Ovalbumin (43 kD), Albumin (67 kD) and Blue Dextran 2000. The SE column run was performed with the Biologic Duo Flow FPLC system from Biorad at 4°C and a flow rate of 1 ml per minute.

LC/MS/MS Identification

Enzymatic Digestion: 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 dithiothreitol and derivatised by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion was carried out overnight at room temperature after an initial two hours incubation at 37°C.

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 lyophilised. Each sample was then resuspended in 23µl of 50 mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex, UK). 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 ionised by electrospray ionisation using a Z-spray source fitted to a QToF-micro (Waters Corp.). 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 analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide.

Database Searching: 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, UK). 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 (S, T and Y), ubiquitination, N-acetylation and glycosylation (raw MS data was check 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 N or C-terminal cleavage of proteins for example. MS/MS sequence information was obtained for all the peptides included in the results.

RESULTS

Purification of the STAT5 protease

We wished to more clearly understand 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 STAT5α to STAT5γ. Previously, it was 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 NP-40 lysis buffer, followed by western blot analysis of lysates using a pan STAT5 antibody. When cells were lysed in boiling SDS-buffer, the majority of STAT5 detected was STAT5 α , and only a small amount of STAT5 γ was detected (Fig. 1A, lanes 1-3). In contrast, when the lysates were prepared in NP-40 lysis buffer, most of the STAT5 proteins were STAT5 γ (Fig. 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 STAT5a as a substrate, we detected the STAT5 protease activity in the nuclear, but not cytoplasmic extracts of 32D cells (Fig. 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 (Fig. 1C). The STAT5 protease activity was inhibited by 4 mM AEBSF in the *in-vitro* protease cleavage assay (Fig. 1C, lanes 2, 4, 6, 8). Furthermore, Stat5a cleavage activity was clearly detected using low amounts of cell extracts (5 μ g-0.625 μ g, lanes 1, 3, 5 and 7), and also in the presence of 4mM 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 1B, 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 eluted with 150 mM sodium chloride from the DEAE column (Fig. 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 hour 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 (Fig. 2B, lane 7). The silver stain gel of the eluted fractions, including the active fractions at 350 and 450 mM NaCl did not clearly reveal specific proteins (lanes 4-11). However, after a 15-fold concentration of the 450 mM fraction, two bands were visible between the 22 kD and 36 kD protein marker bands in a SDS-gel, after silver staining (Fig. 2B, lane 12). The exact size of these bands was determined by size exclusion chromatography, which yielded a single

peak with a light shoulder and a calculated MW of about 23.5 kD and 25.5 kD (data 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 corresponds 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 (data not shown), indicating that they both contain 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% (Fig. 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 (data not shown). From the different parameters used to search the data, there is no obvious explanation as to why the protein resolves as a doublet.

Cathepsin G is responsible for the proteolytic conversion of STAT5

We incubated N-terminal tagged FLAG-STAT5a with nuclear extracts or varying amounts of purified CatG to verify that CatG was the protease that cleaves STAT5 in 32D cells. The resulting STAT5 isoforms were analysed by immunoblotting with anti-FLAG antibody (Fig. 3A). As shown in Figure 3A, STAT5 γ generated by purified CatG is of the same size as STAT5 cleaved by the protease in 32D nuclear extracts (lane 1 vs. 2-4). In contrast, the cleavage pattern of STAT5 generated by purified human Leukocyte Elastase (hLE) appears similar, but the truncated form observed was subtly smaller (lane 1 vs. 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 (Fig. 3B). The protease inhibitor specific for CatG blocks the activity of CatG to cleave STAT5a in the *in-vitro* protease assay (lane 1 and 2), while the specific hLE inhibitor does not block this activity (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 (Fig. 3C, lanes 2-4). By contrast, there was no reduced protease activity in the presence of 250 μ M hLE specific inhibitor (lanes 6 and 7), while even 25 μ M hLE inhibitor can inhibit the

activity of purified hLE (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 (Fig. 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 hour at 37°C), but with varying amounts of purified CatG instead of 32D cell nuclear extract. As shown in figure 3D, the amount of FLAG-STAT5a (α or γ) detected was strongly reduced with increasing amounts of CatG added (lanes 2-4) and was completely undetectable in the presence of 10 mU CatG (lane 1). This indicates 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 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 is due to an authentic *in-vivo* biologic processing event or instead due 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 4mM AEBSF or the specific CatG inhibitor in all of the buffers. In contrast to AEBSF, which inhibits multiple serine proteases, the CatG inhibitor allowed us to specifically block CatG activity only. We also included one sample of nuclear extract prepared in boiling SDS-buffer, in which all proteases are denatured. As shown in Fig. 4, in both 32D and FDC-P1 cells, only STAT5 α was detected by western blotting for STAT5 when the extracts were prepared in the presence of the specific CatG inhibitor (lanes 3 and 8). By contrast, the nuclear extracts prepared without inhibitors (lanes 1 and 5) or with the serine protease inhibitor AEBSF alone (lanes 2 and 6) contained predominantly STAT5 γ . Furthermore, the addition of AEBSF and CatG specific inhibitor together did not increase the amount of STAT5 α detected over the addition of the CatG-specific inhibitor alone (lane 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 1C, 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-buffer (lane 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 to 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 leukemia 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 enhance our understanding of the regulation of STAT proteins in normal hematopoiesis and its potential dysregulation in abnormal malignant hematopoiesis. Therefore, we decided to study this regulation in normal myeloid cell differentiation.

Previously, it was shown that STAT5 γ is generated from the full-length form by limited proteolysis [18, 20, 22, 23, 34]. The responsible STAT5 protease 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 characterised and shown to be sensitive to PMSF, a broad-spectrum serine protease inhibitor, but not to aprotinin, pepstatin, benzamidine [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 kD, and activity was observed in a wide range of physiological pH values. The cleavage site in murine STAT5 was mapped between Y720 and M721 in STAT5a and Y724 and M725 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 (data not shown).

Together with leukocyte elastase, proteinase 3 (PR3) and azurocidine, a proteolytically inactive protease homolog, CatG belongs to a superfamily of azurophil 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 hematopoietic 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 kD and 25.5 kD, because both bands were precipitated with the serine protease inhibitor AEBSF coupled to beads, while CatG was the only protein identified by mass-spectroscopy. To exclude the possibility that other serine proteases from the azurophile granule are also 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 that case, CatG is only a contamination 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 leukemic cell line NB4, Biggs et. al [40] demonstrated that the human brm 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, but 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 also 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 1C. But also in this *in-vitro* cleavage assay, where the nuclear extract was 20x diluted in the sample mix (lane 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-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-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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TABLE OF ABBREVIATIONS

CM, carboxymethyl

DEAE, diethylaminoethanol

DMEM, Dulbecco's Modified Eagle Medium

LC, liquid chromatography

MS, mass spectrometry

m/z, mass-to-charge-ratio

RPMI, Roswell Park Memorial Institute

FIGURE LEGENDS

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

(A) 32D cells were lysed in equal volumes of hot SDS-sample buffer (lanes 1-3) or conventional NP-40 lysis-buffer (lanes 4-6). Proteins of 10, 5 and 1 μ l of the lysates were separated on an SDS gel and the STAT5 proteins were analysed by immunoblotting using anti-pan STAT5 antibody, detecting full-length and C-terminal truncated STAT5. (B) 25 μ g cellular (lane 1) or 5 μ g nuclear (lane 2) extracts from 32D cells were mixed with FLAG-STAT5a (293T cell lysate, 5 μ g) in a total volume of 40 μ l and incubated for 15 min. at 37°C. 20 μ l were separated on an SDS-gel followed by immunoblotting with anti-FLAG antibody. As control, FLAG-STAT5a-containing lysate were incubated for 15 min. at 37°C alone (lane 3). (C) Varying amounts of 32D cell nuclear extracts were incubated with FLAG-STAT5a (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 on an SDS-gel followed by immunoblotting with anti-FLAG antibody. As control, FLAG-STAT5a-containing lysate were incubated for 20 min. at 37°C alone (lane 9).

Figure 2. Purification of the STAT5 protease.

To assess progress during purification of the STAT5 protease activity, 23 μ l of each fraction from the ion exchange columns were separated by 12% SDS-PAGE 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 293T cell lysates containing FLAG-STAT5a and incubated for 60 min. at 37°C, followed by immunoblotting with anti-FLAG antibody. **(A)** Proteins separated after the DEAE-column, silver staining (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 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 15 fold concentrated and 23 μ l were separated in a 16 % SDS-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) (VSP)) was identified as a single protein in both bands analysed by MS. The sequence coverage was 56 % and matched peptides are shown in bold.

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

(A) 2.5 μ g nuclear extract (lane 1) or varying amounts of CatG (lanes 2-4) or hLE (lanes 5-7) were mixed with 1 μ l 293T cell lysate containing FLAG-STAT5a in a total volume of 20 μ l and incubated for 20 min. at 37°C, followed by immunoblotting with anti-FLAG antibody. As control, the 293T cell lysate was incubated for 20 min. at 37°C alone (lane 8). **(B)** The STAT5 protease is inhibited by a protease inhibitor specific for CatG but not by an hLE inhibitor in the *in-vitro* cleavage assay. 2.5 mU CatG were mixed with 1 μ l 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°C, followed by immunoblotting with anti-FLAG antibody. As control, the 293T cell lysate was incubated for 30 min. at 37°C alone (lane 4). **(C)** 2.5 μ g 32D cell nuclear extract were mixed with 1 μ l 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) (lane 2-4) or 250 μ M of the hLE inhibitor (lane 7) and incubated for 30 min. at 37°C, followed by immunoblotting with anti-FLAG antibody. As control, the 293T cell lysate was incubated for 30 min. at 37°C alone (lanes 5 and 10). The protease activity in the 32D nuclear extract was blocked in the presents of 250 μ M of the CatG inhibitor, whereas the addition of an hLE inhibitor showed no effect. **(D)** Varying amounts of CatG (lanes 1-4) were mixed with 1 μ l 293T cell lysate containing FLAG-STAT5a in a total volume of 20 μ l and incubated for 60 min. at 37°C, followed by immunoblotting with anti-FLAG antibody. As control, the 293T cell lysate was incubated for

60 min. at 37°C alone (lane 5).

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

20 µl nuclear extracts prepared from 32D (left panel) or FDC-P1 cells (right panel) were separated by SDS-PAGE followed by western blotting using 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 µM CatG inhibitor (lane 7) or with 250 µM 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 hot SDS-buffer (lanes 4 and 9). The full-length and truncated forms of STAT5 are indicated with arrows.

Figure 1

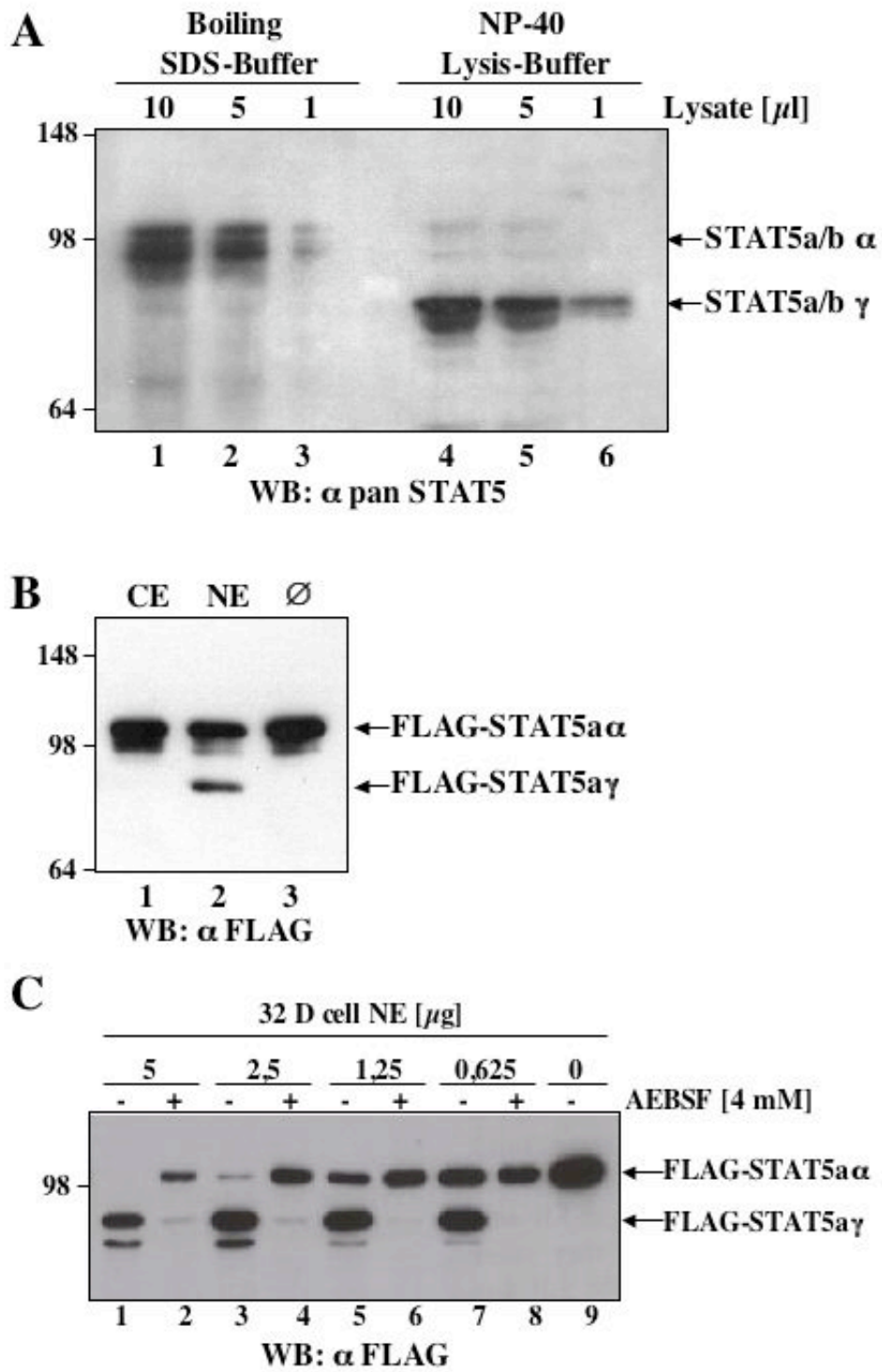


Figure 2

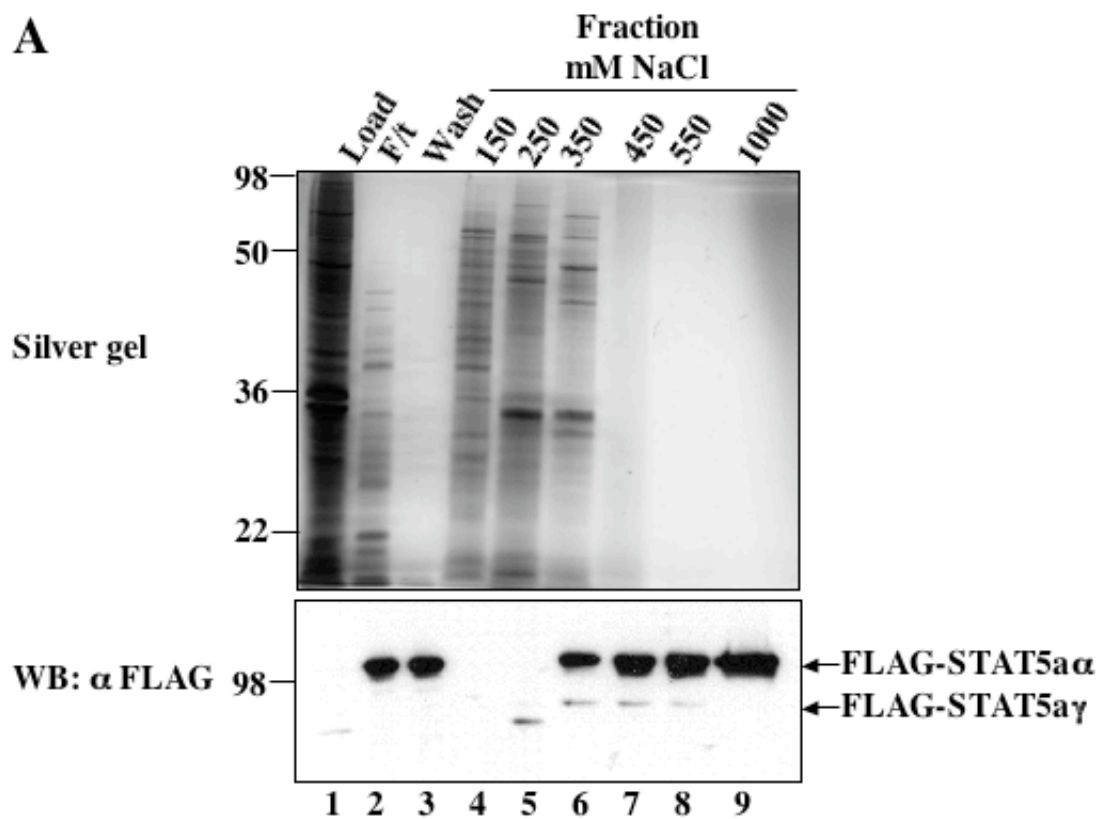
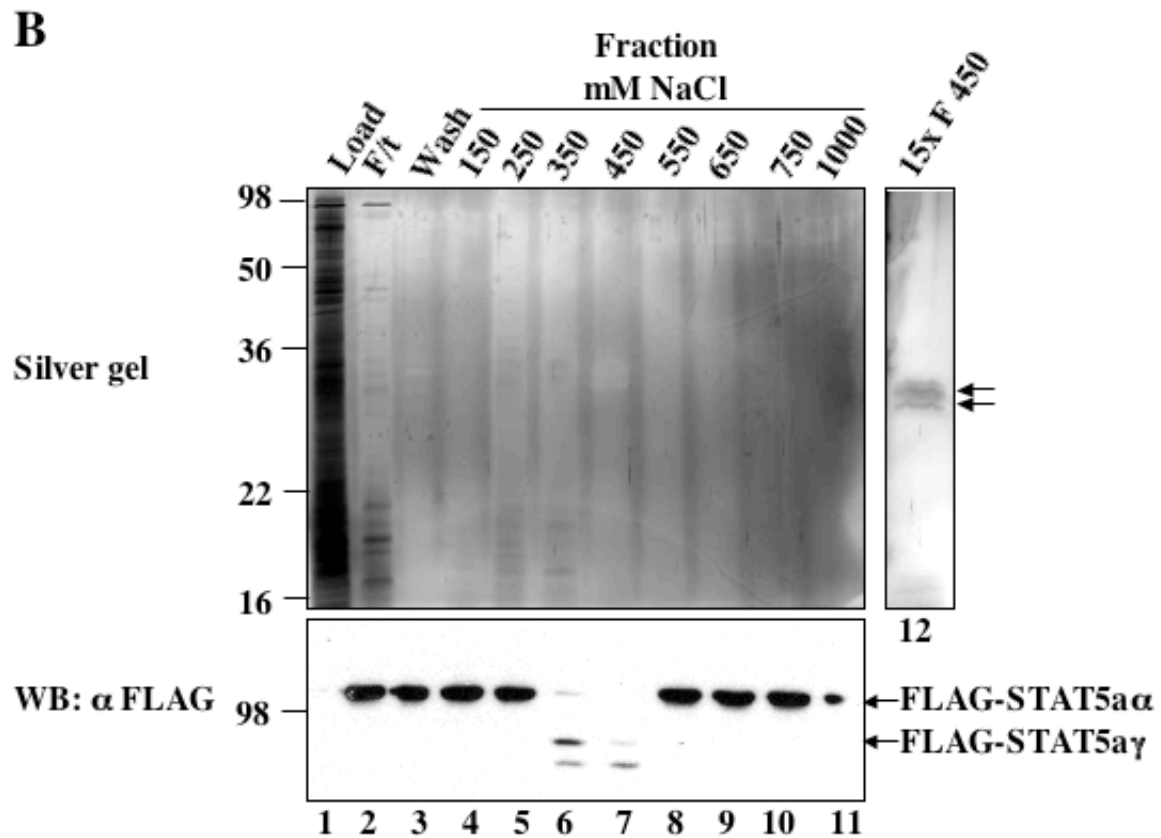


Figure 2



C

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151 SQRSGTNVLQ EVQLRVQMDQ MCANRFQFYN SQTQICVGNP RERKSAFRGD
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Figure 3

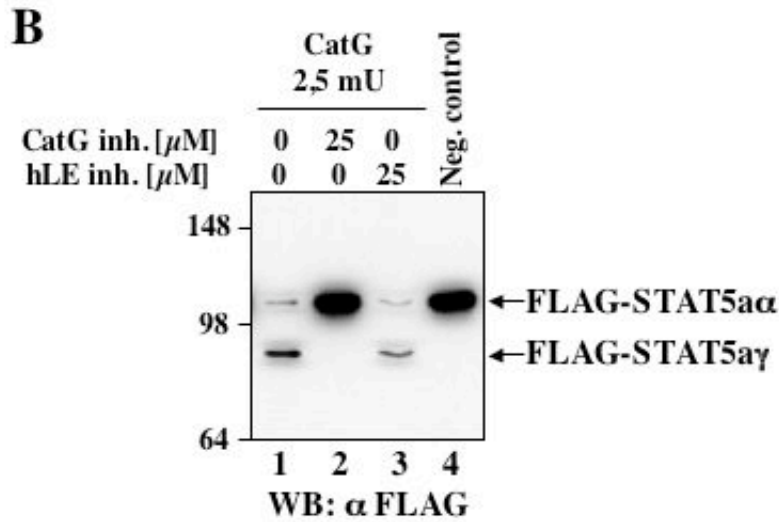
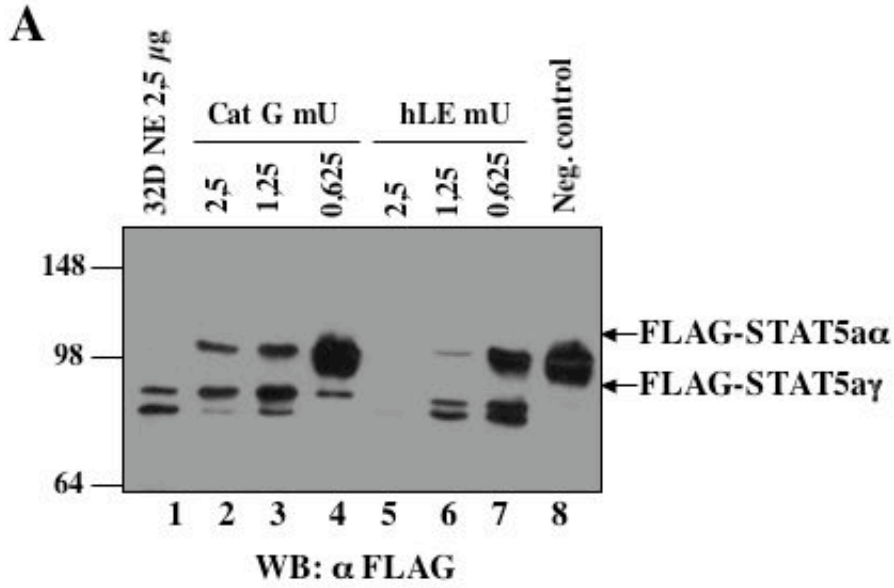


Figure 3

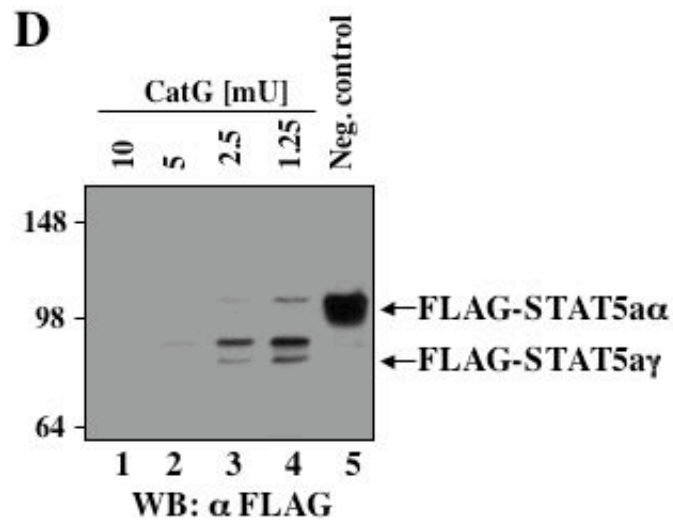
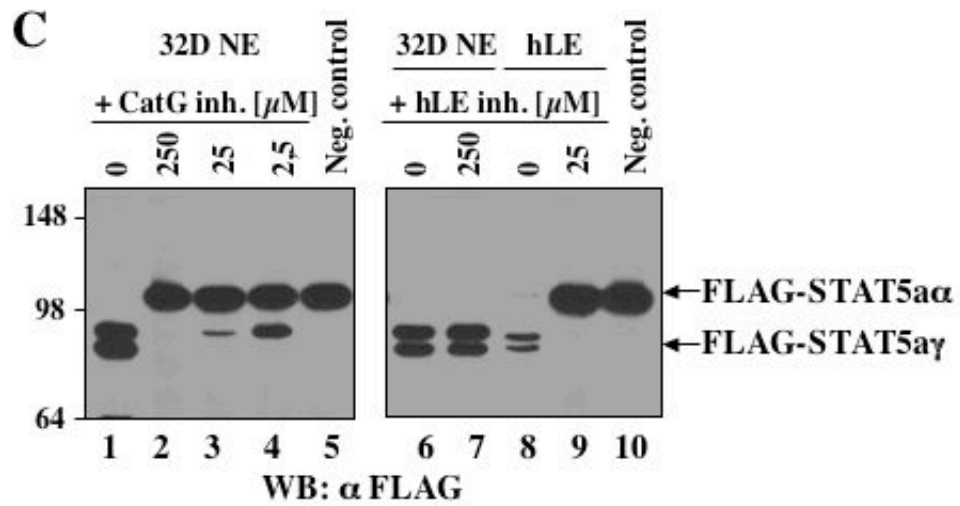


Figure 4

