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Interleukin-3 signals through multiple isoforms of Stat5

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The interleukin (IL)-3 family of cytokines mediates its numerous effects on myeloid growth and maturation by binding a family of related receptors. It has been shown recently that IL-3 induces the activation of two distinct cytoplasmic signal transducing factors (STFs) that are likely to mediate the induction of immediate early genes. In immature myeloid cells, IL-3 activates STF–IL-3a, which comprises two tyrosine-phosphorylated DNA binding proteins of 77 and 80 kDa. In mature myeloid cells, IL-3 and granulocyte-macrophage colony-stimulating factor activate STF–IL-3b, which consists of a 94 and 96 kDa tyrosine-phosphorylated DNA binding protein. Peptide sequence data obtained from the purified 77 and 80 kDa proteins (p77 and p80) indicate that they are closely related but are encoded by distinct genes. Both peptide and nucleotide sequence data demonstrate that these two proteins are the murine homologs of ovine mammary gland factor (MGF)/Stat5. The peptide data also indicate that p77 and p80 are phosphorylated on tyrosine 699, a position analogous to the tyrosine that is phosphorylated in Stat1 and Stat2 in response to interferon. Additionally, antiserum raised against bacterially expressed p77/p80 recognizes the 94 and 96 kDa protein components of STF–IL-3b, suggesting that these may be additional isoforms of Stat5. These studies indicate that the IL-3 family of ligands is able to activate multiple isoforms of the signal transducing protein Stat5.

Key words: GM-CSF/IL-3/signal transducing factor/Stat5

Introduction

Cytokines regulate multiple aspects of hematopoiesis through their interaction with receptors from the cytokine superfamily. Upon binding ligand, these receptors stimulate specific intracellular signals, many of which culminate in altered gene expression. Until recently, the nature of the molecules that mediate the rapid transduction of these signals to the nucleus has been poorly understood. However, a detailed analysis of the ability of interferons (IFNs) to activate genes has led to the identification of a new signaling paradigm, the JAK kinase–signal transducers and activators of transcription (STAT) protein pathway. In the case of IFN-α, two tyrosine kinases, Jak1 and tyk2, and three STAT proteins, Stat1–3, transduce signals from the receptor to the nucleus (Schindler *et al.*, 1992b; Velazquez *et al.*, 1992; Müller *et al.*, 1993; Darnell *et al.*, 1994; unpublished observation). In contrast, IFN-γ signals through the activation of Jak1, Jak2 and Stat1 (Shuai *et al.*, 1992; Müller *et al.*, 1993; Silvennoinen *et al.*, 1993; Watling *et al.*, 1993; Darnell *et al.*, 1994). Recently, a number of other cytokines have been shown to signal through this paradigm. For example, the interleukin (IL)-6 family of ligands [e.g. IL-6, IL-11, ciliary neurotrophic factor (CNTF), oncostatin M (OnM) and leukemia inhibitory factor (LIF)], which employs a common gp130 receptor component, signals through gp130-associated JAK kinases, Stat1 and Stat3 (Bonni *et al.*, 1993; Akira *et al.*, 1994; Lütticken *et al.*, 1994; Stahl *et al.*, 1994; Wegenka *et al.*, 1994; Zhong *et al.*, 1994). Additionally, IL-4 has been shown recently to signal through Jak1, Jak3 and Stat6 (Campbell *et al.*, 1994; Hou *et al.*, 1994; Witthuhn *et al.*, 1994), and prolactin (PRL) has been shown to signal through Jak2 and Stat5 [mammary gland factor (MGF)] in ovine mammary gland tissue (Campbell *et al.*, 1994; DaSilva *et al.*, 1994; Dusanter-Fourt *et al.*, 1994; Wakao *et al.*, 1994). In each case, these signals appear to mediate the activation of immediate early genes, which play an important role in the ensuing biological response.

The IL-3 family of ligands, which includes IL-3 (multicolony-stimulating factor), IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), also promotes the rapid activation of genes (Kishimoto *et al.*, 1994; Quelle *et al.*, 1994) and appears to signal through the JAK–STAT pathway. These ligands play an important role in the maturation, proliferation and activation of myeloid lineages. Of this group, the spectrum of activity of IL-5 is most restricted, playing an important role in the development and activation of eosinophils, basophils and mast cells. 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, IL-3, IL-5 and GM-CSF bind a family of 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. In the murine system, there is an additional IL-3-specific β-chain (Sakamaki *et al.*, 1993). The β-chain has been shown to stimulate a number of

overlapping intracellular signals, including the tyrosine phosphorylation of a similar set of proteins in response to each of these ligands (Sorensen *et al.*, 1989; Murata *et al.*, 1990; Duronio *et al.*, 1992; Quelle *et al.*, 1993; Kishimoto *et al.*, 1994; Matsuguchi *et al.*, 1994). One of these phosphoproteins is the protein tyrosine kinase Jak2. Detailed studies have identified the membrane-proximal region of the β-chain as being important for the interaction with and activation of Jak2 (Quelle *et al.*, 1994). This leads to the subsequent activation of STAT proteins, which associate and form active signal transducing factors (STFs).

Recent studies from our laboratory have determined that IL-3 stimulates two different STFs, depending on the differentiation state of the target cell. In immature myeloid cells STF−IL-3a is activated, while in mature myeloid cells a biochemically distinct STF, STF−IL-3b, is activated (Rothman *et al.*, 1994). These STFs are activated by tyrosine phosphorylation, rendering them competent to bind to the IFN−gamma activation sites (GAS) family of enhancer elements (Pellegrini and Schindler, 1993). The ability of STAT-like components of STFs to bind GAS-related elements appears to be critical for the JAK−STAT signaling by most cytokines (Larner *et al.*, 1993; Rothman *et al.*, 1994; Schindler *et al.*, 1994; Zhong *et al.*, 1994). Consistent with this observation, we have demonstrated recently that STF−IL-3a also consists of two tyrosine-phosphorylated GAS binding proteins of 77 and 80 kDa. A similar analysis of STF−IL-3b has identified a 94–96 kDa tyrosine-phosphorylated GAS binding protein(s). Furthermore, the 94–96 kDa protein(s) of STF−IL-3b have been shown to crossreact with a Stat1-derived antibody (ab4; Rothman *et al.*, 1994; unpublished observation). These previous studies have suggested that the proteins identified in STF−IL-3a and STF−IL-3b were likely to be members of the STAT family.

These initial studies have now been extended to include a larger panel of myeloid cells and the related cytokine, GM-CSF. STF−IL-3a is shown to be activated by IL-3 in several immature myeloid cell lines, while STF−IL-3b is activated by IL-3 and GM-CSF in several more mature myeloid cells. In an effort to determine whether these signaling factors are related, the components of STF−IL-3a have been purified and cloned. Peptide sequences derived from purified p77 and p80 demonstrate that these proteins are encoded for by closely related but distinct genes, which have an ~80% amino acid identity with ovine MGF/Stat5 (Wakao *et al.*, 1994). Furthermore, a detailed analysis of these peptide sequences indicates that tyrosine 699 (Y699) is phosphorylated. This tyrosine is in a position that is analogous to the position of the tyrosine that is phosphorylated in a ligand-dependent manner in Stat1 and Stat2 (Shuai *et al.*, 1993; Improta *et al.*, 1994). Additionally, antibodies generated against these proteins crossreact with the 94–96 kDa protein(s) identified in STF−IL-3b, suggesting that these proteins are also related to Stat5.

Results

Comparison of STF induced by IL-3 and GM-CSF

Previously we have shown that IL-3 can stimulate two different GAS binding activities, STF−IL-3a and STF−IL-3b. STF−IL-3a, which is activated in immature cells, has a faster mobility on an electrophoretic mobility shift assay (EMSA). STF−IL-3b, which is activated in more mature myeloid cells, has a substantially slower mobility and shares several features with STF−IL-2, STF−IL-7 and STF−erythropoietin (Epo; data not shown). In an effort to characterize further the correlation between the IL-3 family ligands and the STFs they activate, we examined several additional cell lines. As shown in Figure 1A, several early myeloid lineages, including DA-3(Epo1), 32D and FDC-P1 (FDtrk; see below), activate STF−IL-3a in response to IL-3, but not other cytokines (data not shown). DA-3(Epo1) cells are exceptional (as early myeloid cells) in their ability to also respond to Epo. They were derived by transfecting the wild-type Epo receptor into an IL-3-dependent early myeloid cell line (Miura *et al.*, 1991). Although these cells proliferate in response to Epo, they are not induced to differentiate, as is the case for more mature myeloid cells like 32Dc1(Epo) cells (Migliaccio *et al.*, 1989). In more mature myeloid cells, including 32Dc1(Epo), U937 and MOLT4, IL-3 and GM-CSF promote the rapid activation of GAS binding activities that have very similar mobilities on EMSA. The possibility that the STFs activated by IL-3 and GM-CSF are identical is further supported by their reactivity with an antibody made against Stat1 (ab4; see Figure 1B), which has the curious property of not supershifting Stat1 but rather supershifting a STAT-related protein (Rothman *et al.*, 1994; A.Pernis, S.Gupta, J.Yopp, H.Kashleva, C.Schindler and P.Rothman, manuscript submitted). Moreover, the IL-3 and GM-CSF STFs did not react with another crossreactive antibody (ab1), while the IL-6-induced GAS binding activities did react as expected (Rothman *et al.*, 1994). Additionally, both of these STFs have the same pattern of competition with a panel of GAS probes (data not shown), again consistent with identity. These observations are reminiscent of the IL-6 family of ligands which signal through a common receptor and all activate the same STF. In contrast, however, IL-3 is able to activate a distinct STF in immature cells.

Purification of STF−IL-3a

We were intrigued by the unique properties of STF−IL-3a and set out to purify this GAS binding activity. We initially sought a cell line that would be convenient for large-scale purification, and examined FDtrk cells. These cells were obtained by transforming the IL-3-dependent early myeloid cell line, FDC-P1, with the Trk oncogene, rendering it IL-3-independent (Katzav *et al.*, 1989). As indicated in Figure 1, these cells still respond to IL-3 with the induction of STF−IL-3a. We have demonstrated previously that this is bona fide STF−IL-3a (Rothman *et al.*, 1994).

To purify the STF−IL-3a complex, large volumes of FDtrk cells were grown and induced with IL-3. Detergent-free whole-cell extracts were fractionated sequentially by heparin agarose and GAS oligonucleotide affinity chromatography. STF−IL-3a was recovered from the heparin agarose column by elution with a linear NaCl gradient, as indicated in Figure 2A. Peak activity was pooled and fractionated further on a GAS oligonucleotide affinity column by binding in low salt (150 mM) and

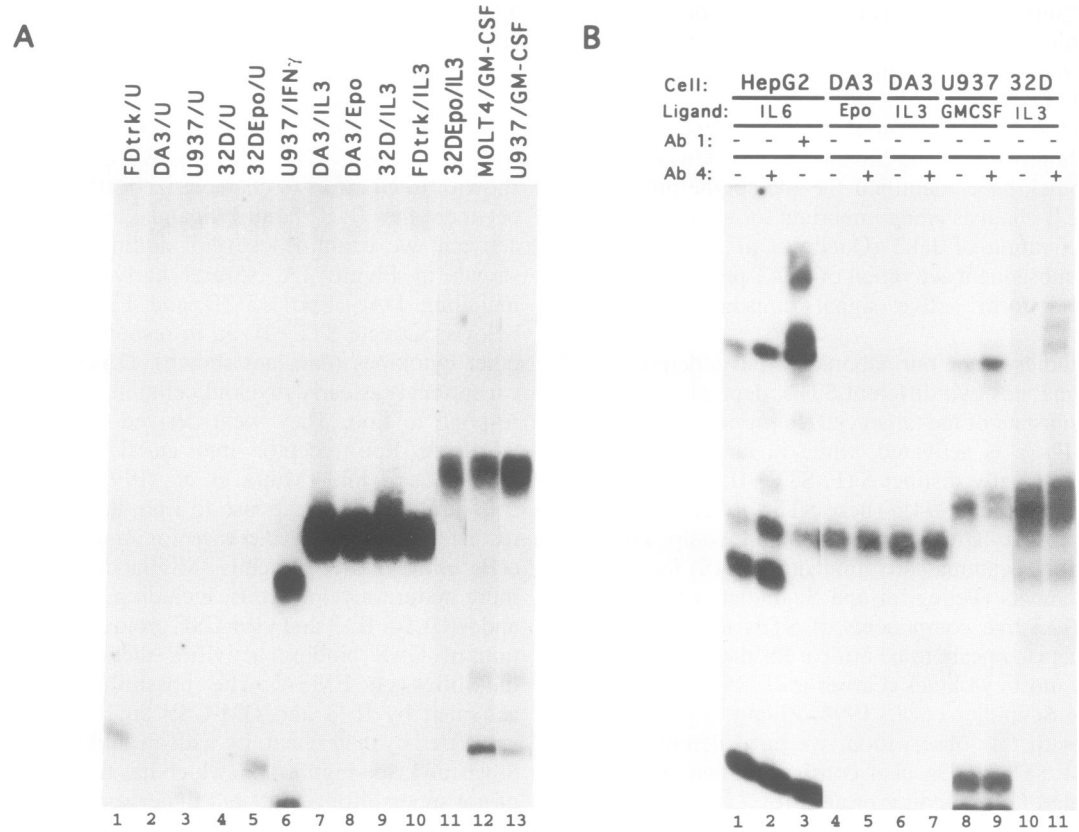

Fig. 1. Mobility shift analysis of cytokine-stimulated GAS binding activities. (**A**) EMSA on different cell extracts. Cytoplasmic, nuclear or whole-cell extracts were prepared from cells before and after stimulation with cytokines, and then examined by EMSA with a labeled IRF1 GAS probe. Lanes 1–5, untreated cells; lane 6, whole-cell extract from IFN-γ-stimulated (15 min) U937 cells; lanes 7 and 8, nuclear extract from IL-3-stimulated (15 min) DA-3(EpoR) cells; lane 9, whole-cell extract from IL-3-stimulated (15 min) 32Dc1 cells; lane 10, whole-cell extract from IL-3-treated FDtrk cells; lane 11, nuclear extract from IL-3-stimulated (15 min) 32Dc1(Epo1) cells; lane 12, nuclear extract from GM-CSF-stimulated (15 min) MOLT4 cells; lane 13, cytoplasmic extract from GM-CSF-stimulated (15 min) U937 cells. (**B**) Antibody interference mobility shift assays of cytokine-activated STFs. Cells were treated and extracts prepared and assayed as described above. EMSAs were performed after the addition of immune (+) or non-immune (−) serum.

elution with 1 M NaCl. The eluted material was examined by silver staining and shown to contain only two proteins of 77 and 80 kDa (Figure 2C). These purified proteins, which were also tyrosine-phosphorylated (Figure 2C), comigrated with the previously characterized p77 and p80 phosphoproteins (data not shown). The ability of these two purified proteins to reconstitute a GAS binding activity identical to STF−IL-3a induced by IL-3 in crude extracts (Figure 2B) provides compelling evidence that these two proteins account for the entire activity of STF−IL-3a.

Analysis of p77- and p80-derived peptides

In an effort to determine the identity of p77 and p80, the purified fractions were resolved on SDS−PAGE, transferred to nitrocellulose and excised for microsequence analysis. Individual p77 and p80 peptides, obtained by trypsin digestion, were fractionated by HPLC. The chromatographic patterns were very similar, consistent with the presence of two highly related proteins (data not shown). The mass spectrometry and sequence analyses of some of the corresponding peptide pairs (e.g. peptides 77T24a and 80T21a in Table I) were consistent with identity. However, other peptide pairs showed subtle differences in their molecular composition/sequence (e.g. peptide pairs 77T23/80T15 and 77T7/80T2 in Table I), excluding the possibility that p77 was a truncated form of p80. When compared with a database, each of the nine p77 and eight p80 peptides sequenced (Table I) exhibited a high degree of identity with ovine MGF/Stat5 (Wakao *et al.*, 1994). Notably, the p77 peptides and the corresponding cDNA sequence (data not shown) indicated that p77 is more closely related to MGF/Stat5 than p80.

Two of the peptides, 77T7 and 80T2, corresponded to the region of Stat1 and Stat2 that is activated by tyrosine phosphorylation (Shuai *et al.*, 1993; Improta *et al.*, 1994). Interestingly, both of these peptides eluted from the reversed-phase column earlier than would have been expected based on their composition and length. Also, during sequencing, tyrosine was not detected during cycle 5, as would have been predicted from the amino acid sequence of MGF/Stat5. Additionally, the difference of 242.24 Da between the theoretical mass (assuming a single gap at position 5) and the experimental mass of peptide 77T7 was too large to be accounted for by a single amino acid. However, the average isotopic mass of phosphotyrosine (243.156 Da) is uniquely close to this difference, indicating that this residue was phosphotyrosine. Furthermore, the presence of a phosphotyrosine at this position would explain the other unusual properties of these peptides, because phosphotyrosines are not detected by standard sequencing procedures and their hydrophobicity promotes earlier elution from a reverse-phase matrix. The

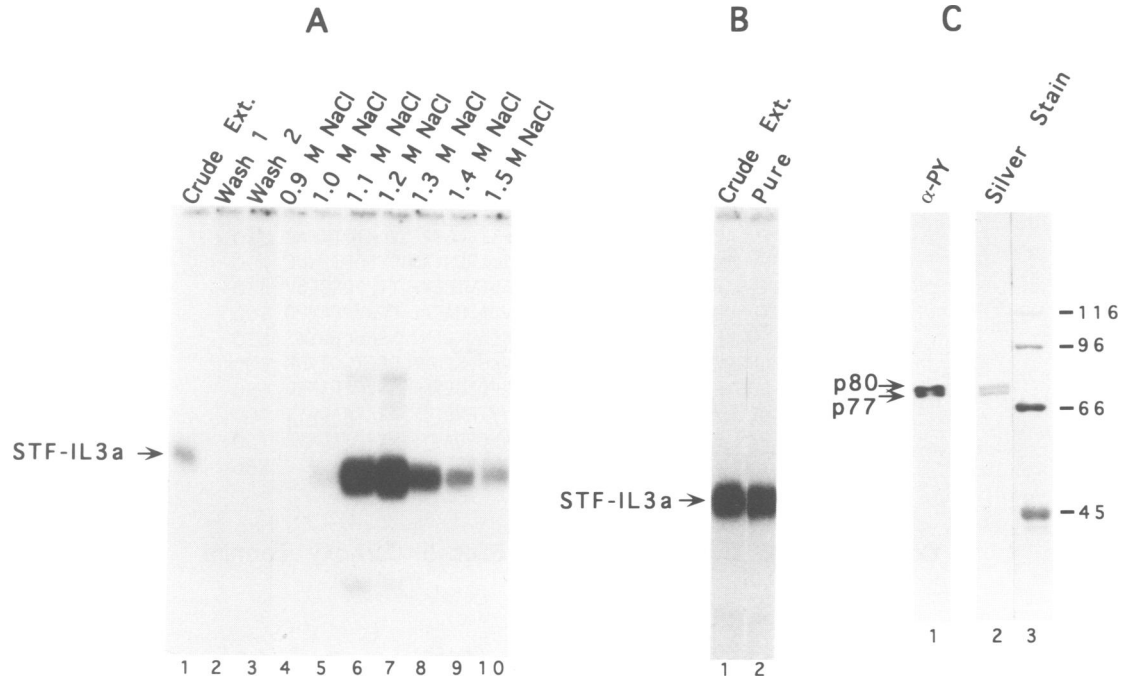

Fig. 2. Purification of STF−IL-3a from FDtrk cell extracts. (**A**) Heparin agarose column chromatography. Proteins from whole-cells extracts of FDtrk cells were fractionated on a heparin agarose column. The column was washed twice and the proteins eluted with a linear NaCl gradient. Eluted fractions were assayed for STF−IL-3a activity by EMSA as described in Figure 1. (**B**) Oligonucleotide affinity chromatography. STF−IL-3a from FDtrk cell crude extract (lane 1) and after oligonucleotide affinity chromatography (lane 2) were compared by EMSA as described in Figure 1. (**C**) SDS−PAGE of the oligonucleotide affinity-purified STF−IL-3a. The purity of the eluted protein was checked by silver staining (Schindler *et al.*, 1992a) and immunoblotting. Samples were fractionated on a 7% SDS−PAGE. Antibody binding was detected by ECL (Amersham). Molecular weight markers (Sigma) are as indicated.

Table I. Amino acid sequence data from the p77- and p80-derived peptides

Peptide	Sequence	*m/z*	[MH$^+$]	Δ	MGF AA (position)
77-T31	QMQVLYGQHFPIEVR	1846.0	1847.17	0.17	
MGF	···				17–31
77-T45	HYLAQxIESQPxDx	n.d.			
80-T42	···x·x··x·Ax				
MGF	···A·W··S·PW·A				32–45
80-T23	HLQINQTFEELR	1528.9	1528.72	0.18	
MGF	···				142–153
77-T24a	IQAQFAQLGQLNPQER	1843.6	1842.07	1.53	
80-T21a	···x				
MGF	···A····E·				183–197
77-T33	QVSLETWLQR	1260.3	1260.43	0.13	
MGF	····A····				209–218
77-T24b	LAEIIWQNR	1143.9	1143.33	0.57	
80-T21b	····x··x				
MGF	····W··R				282–289
77-T50	TLxLPVVVIVx	n.d.			
80-T49	··x·····x				
MGF	··S·····H				461–471
77-T24c	ENLVFLAQK	n.d.			
80-T21c	·····				
MGF	···L····				528–536
77-T7	AVDGxVKPQIK	1297.5	1055.26	242.24	
80-T2	·A··x····x				
MGF	·V··Y····K				690–700
77-T23	QVVPEFVNAxTDx	n.d.			
80-T15	·····A··x				
MGF	·····VS·SADS				701–713

Alignment of p77 and p80 tryptic peptides with MGF/Stat5 sequences. Sequences of tryptic peptides were obtained by combined Edman−chemical/mass spectrometric analysis; MGF sequences were taken from Wakao *et al.* (1994). x denotes that no amino acid could be identified at this position; dots indicate that the residue in this position is identical to the one in the top line of that particular set. *m/z* values were obtained experimentally by MALDI mass spectrometry; [MH$^+$] is the theoretical average isotopic mass (M) summed from the identified amino acids, plus one proton [H$^+$]. Δ is the difference between experimental and theoretical mass values. The final column lists the positions in the published MGF sequences shown.

A Amino Acid Sequence of p80

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MAMWIQAQQL QGDALHQMQA LYGQHFPIEV RHYLSQWIES QAWDSIDLDN  50
PQENIKATQL LEGLVQELQK KAEHQVGEDG FLLKIKLGHY ATQLQSTYDR 100
CPMELVRCIR HILYNEQRLV REANNGSSPA GSLADAMSQK HLQINQTFEE 150
LRLITQDTEN ELKKLQQTQE YFIIQYQESL RIQAQFAQLG QLNPQERMSR 200
ETALQQKQVS LETWLQREAQ TLQQYRVELA EKHQKTLQLL RKQQTIILDD 250
ELIQWKRRQQ LAGNGGPPEG SLDVLQSWCE KLAEIIWQNR QQIRRAEHLC 300
QQLPIPGPVE EMLAEVNATI TDIISALVTS TFIIEKQPPQ VLKTQTKFAA 350
TVRLLVGGKL NVHMNPPQVK ATIISEQQAK SLLKNENTRN DYSGEILNNC 400
CVMEYHQATG TLSAHFRNMS LKRIKRSDRR GAESVTEEKF TILFDSQFSV 450
GGNELVFQVK TLSLPVVVIV HGSQDNNATA TVLWDNAFAE PGRVPFAVPD 500
KVLWPQLCEA LNMKFKAEVQ SNRGLTKENL VFLAQKLFNI SSNHLEDYNS 550
MSVSWSQFNR ENLPGRNYTF WQWFDGVMEV LKKHLKPHWN DGAILGFVNK 600
QQAHDLLINK PDGTFLLRFS DSEIGGITIA WKFDSQERMF WNLMPFTTRD 650
FSIRSLADRL GDLNYLIYVF PDRPKDEVYS KYYTPVPCEP ATAKAADGYV 700
KPQIKQVVPE FANASTDAGS GATYMDQAPS PVVCPQAHYN MYPPNPDSVL 750
DTDGDFDLED TMDVARRVEE LLGRPMDSQW IPHAQS               786
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B Comparison of Ovine and Murine Stat 5 Carboxy Termini

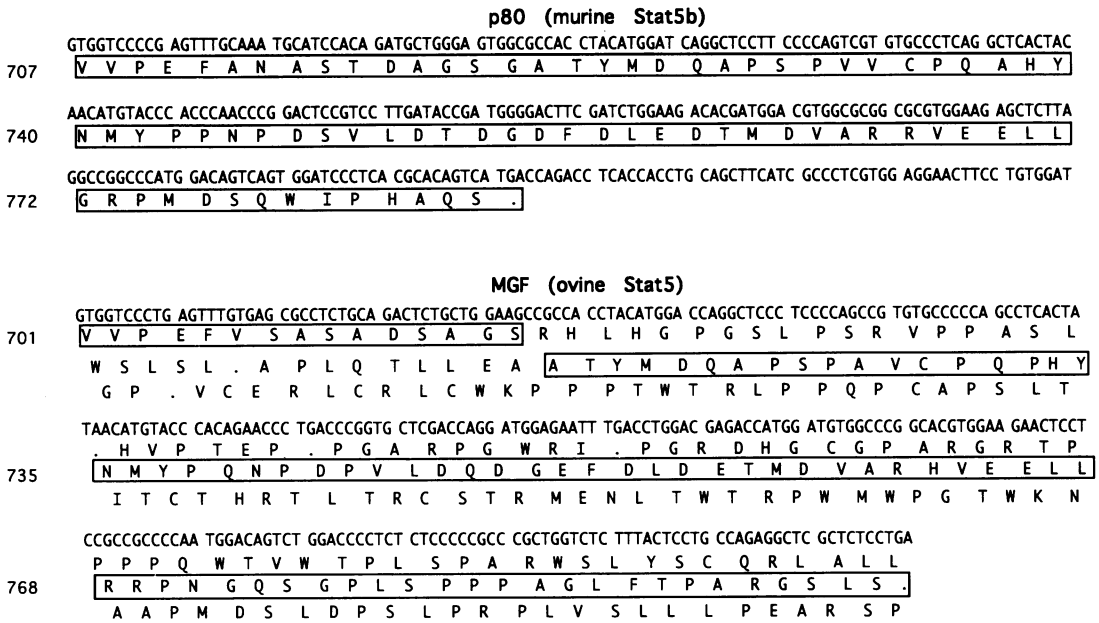

Fig. 3. p80 encodes a 786 amino acid protein that is related to MGF (Stat5). (**A**) Conceptually translated amino acid sequence of p80. The predicted p80 protein sequence encoded by the longest open reading frame (ORF) is shown. At 3 bp upstream from the initiator methionine residue of this ORF there is a translation termination codon, consistent with the interpretation that the upstream sequences are untranslated. Amino acid sequences obtained from the microsequencing of the purified p80 are underlined. (**B**) C-terminal sequence comparison between murine p80 and MGF. Boxed regions in the upper panel show the C-terminus of the p80 sequence derived from the conceptually translated amino acid sequence, as shown above. The lower panel demonstrates amino acid translation in all three reading frames of the MGF sequence. Boxed regions illustrate a shift in reading frames in the amino acid homology between MGF and p80.

presence of tyrosine at this position in p77 and p80 has now also been confirmed by nucleotide sequencing of the corresponding cDNA clones (see below). These results establish that Y699 is phosphorylated in both purified proteins. In addition, these results indicate that p77 and p80 are encoded for by two distinct genes, both of which appear to be the murine homologs of Stat5.

Cloning of p77 and p80

The availability of partial amino acid sequence data allowed us the opportunity of cloning the corresponding cDNAs. A pair of degenerate primers, based on the amino acid sequence of peptide 77T23 and a highly conserved sequence in the STAT SH2 domain (GTFLLRF; e.g. Wakao *et al.*, 1994), amplified the anticipated 300 bp PCR product from a murine thymocyte cDNA library. The PCR product was then employed to screen a thymocyte and murine mast cell library. Sequence analysis of the identified clones indicated that a majority of cDNAs encoded p80, while two clones encoded p77. A 4.5 kb p80 cDNA was obtained by combining two overlapping clones. This clone, which has a poly(A) tail, is likely to be full length because

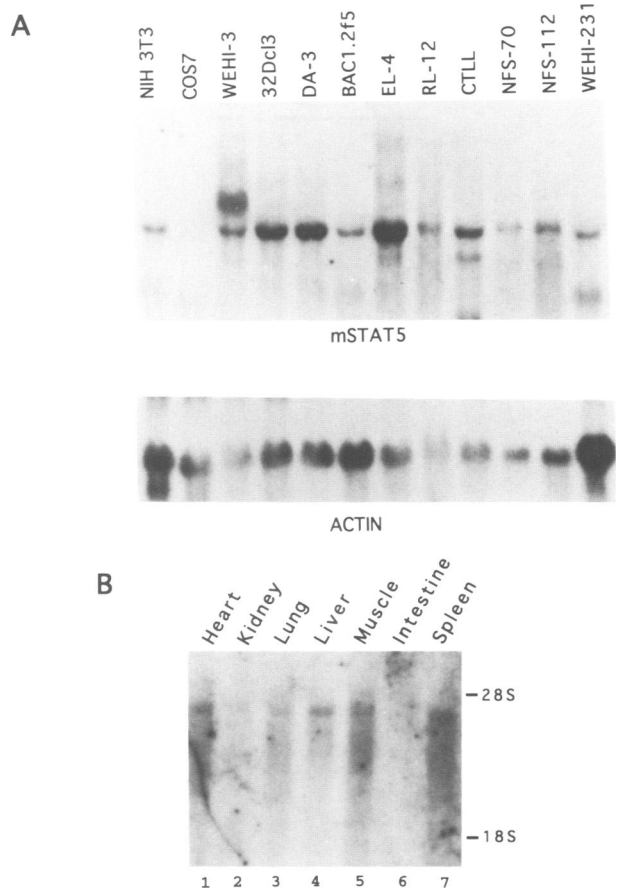

Fig. 4. Northern blot analysis of p77 and p80 clones. (**A**) Northern blot of cell lines. 20 μg of total RNA from different cell lines (as indicated) were separated by denaturing gel electrophoresis and transferred onto a nylon membrane. The RNA blot was probed with a radiolabeled 300 bp PCR fragment and then reprobed with actin probe for quantitation. (**B**) Northern blot of mouse tissues. 10 μg of total RNA were prepared from murine tissues as indicated, and fractionated and probed as above. The positions of 28S and 18S rRNA are indicated.

its size corresponds to the size predicted by Northern blotting (see Figure 4). Conceptual translation of the p80 clone indicated that it encoded a 786 amino acid protein, with a predicted molecular weight of 90 kDa (Figure 3A) and an 81% identity (amino acid) with MGF/Stat5. A comparison of the p80 sequence with the peptide data (see Table I) demonstrates that each of the p80 peptide sequences are represented. Furthermore, peptides that are unique to p77 are only encoded for by the p77 cDNA (data not shown), which has 90% identity with the p80 cDNA.

A more detailed comparison of the p80 sequence (derived from three independent clones) with the MGF/Stat5 sequence identifies a small but significant difference in the 3′ coding sequence, leading to a frame shift (Figure 3B). Whereas the amino acid homology ends at p80 amino acid 721 (MGF/Stat5 amino acid 715), the homology at the nucleotide level extends an additional 170 bp (~55 amino acids). When additional MGF/Stat5 reading frames are examined, however, the amino acid sequence homology actually continues for another 55 amino acids. These observations are most consistent with an error in the MGF/Stat5 sequence. In summary, the data in this section concur with the peptide data, in that there are two murine homologs of Stat5.

Expression patterns of p77 and p80

To examine the expression pattern of the murine Stat5 homologs, the 300 bp PCR probe was employed in a series of Northern blot studies. This probe, which is derived from a conserved region (amino acids 613–711), would be anticipated to detect transcripts corresponding to both Stat5 genes. In most tissues this probe detected a single mRNA species of 4.5 kb (Figure 4A). The 4.5 kb message was expressed to a varying degree in all myeloid cells examined. It was also detected at low levels in 3T3 cells, but not in control COS cells. Additional prominent hybridizing bands were detected in WEHI-3, WEHI-231 and CTLL cells. The nature of these extra bands remains to be elucidated. Further studies on RNA prepared from tissues indicated good expression in liver, spleen and muscle (i.e. skeletal and cardiac), consistent with the results published previously on ovine MGF/Stat5 (Wakao *et al.*, 1994). These data indicate that murine Stat5 is expressed in many tissues, including those in which STF−IL-3a and STF−IL-3b activities have been detected. However, in many tissues where both p77 and p80 (and p94 and p96) are expressed, only a single mRNA species is detected. Since it is unlikely that our probe would have failed to detect either p77 or p80 mRNAs, these data suggest that either one of the messages is expressed at a low abundance or that both messages are the same size.

Murine Stat5 antibody detects p77, p80 and p94–96

A polyclonal antiserum raised against a GST fusion protein encoding the 300 bp PCR product was generated to examine whether the components of STF−IL-3a might be related to the components of STF−IL-3b. The antiserum (abSt5) was initially characterized in a Western blot assay. abSt5 recognized p77 more avidly than p80 in both purified fractions and crude extracts (Figure 5A). The identity of the detected p77 and p80 bands was confirmed by silver staining (data not shown) and by reprobing the filters with an antiphosphotyrosine antibody (Figure 5B). These data demonstrated that equimolar quantities of p77 and p80 were present. Additionally, the 94–96 kDa proteins, which can be oligoprecipitated from IL-3-stimulated 32Dc1(Epo) cells (Rothman *et al.*, 1994), were examined with these antisera. The antiphosphotyrosine blot demonstrated that a doublet, consisting of a 94 and 96 kDa protein, was oligoprecipitated from these IL-3-stimulated extracts (Figure 5B). These observations build on our previous studies on the same extracts, in which we identified the same non-specific tyrosine-phosphorylated band of 120 kDa and a broad IL-3-induced band of ~96 kDa (Rothman *et al.*, 1994). However, with an improved protocol we have reproducibly resolved this broad band into the 94 and 96 kDa doublet, as indicated in Figure 5A and B. Similar to that which was observed with p77 and p80, the faster migrating partner of the 94/96 kDa doublet was more avidly recognized by abSt5. Additionally, the non-specific protein (that present in unstimulated extracts) was also recognized by abSt5, suggesting that these proteins may all be related. The

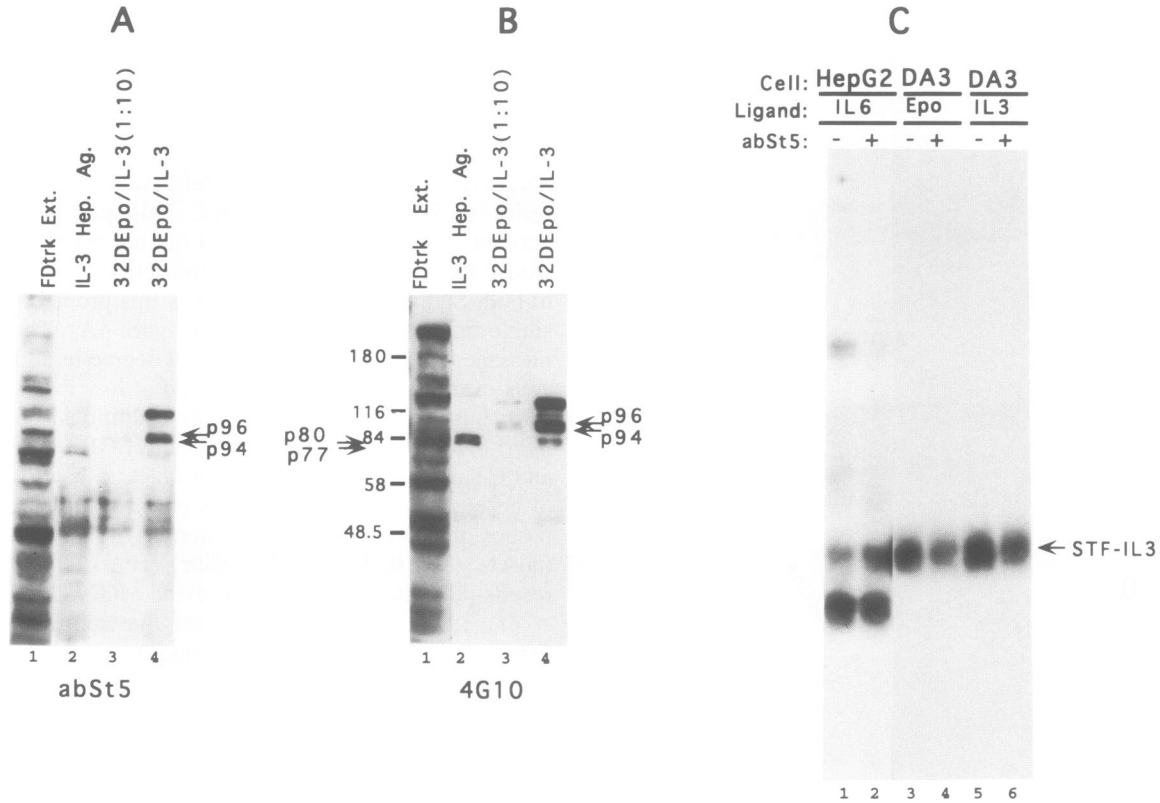

Fig. 5. Characterization of the abSt5 antibody. (**A**) Oligonucleotide affinity precipitations probed with abSt5. Cells were treated and extracts were prepared as described in Figure 1. Oligonucleotide precipitations of IL-3-treated 32Dc1(Epo) cytoplasmic extracts were carried out as described previously (Rothman *et al.*, 1994). Lane 1, crude extract from IL-3-stimulated FDtrk cells; lane 2, heparin agarose partially purified STF−IL-3a (see Figure 2); lanes 3 and 4, oligonucleotide precipitations of IL-3-stimulated 32Dc1(Epo) cells (lane 3 represents a 10-fold dilution of lane 4). Samples were prepared and analyzed as described in Figure 2. abSt5 was used at a 1:200-fold dilution. (**B**) Oligonucleotide affinity precipitations probed with an antiphosphotyrosine antibody. The blot in (A) was stripped and reprobed with antiphosphotyrosine antibody (4G10; UBI). Molecular weight markers (Sigma) are as indicated. (**C**) Antibody interference mobility shift assays with abSt5. Cells were treated and extracts were prepared as described in Figure 1. EMSAs were performed after the addition of abSt5 (+) or non-immune serum (−), as indicated.

ability of abSt5 to recognize these proteins in crude extracts was less obvious.

Next, abSt5 was tested in an EMSA. IL-3-stimulated DA-3 and 32Dc1(Epo) extracts were shifted either before or after the addition of abSt5. As shown in Figure 5C, the antibody partially abrogated the shift complexes in immature cells (i.e. STF−IL-3a). However, the GAS binding complexes in extracts of IL-6-stimulated HepG2 cells were not affected, indicating that the antiserum does not non-specifically affect binding. The data presented in this section demonstrate that abSt5 recognizes the proteins purified from STF−IL-3a, as well as the 94 and 96 kDa proteins that have been identified in STF−IL-3b. They also suggest that p77 and p94 may be more closely related to each other.

Discussion

In this study we demonstrate that IL-3 and GM-CSF activate components of the JAK−STAT pathway which was first elucidated in the IFN system (Pellegrini and Schindler, 1993; Darnell *et al.*, 1994). IFN-α has been shown to activate two distinct JAK−STAT pathways. The activation of genes with IFN-stimulated response elements (ISRE) enhancers is mediated by STF−IFNα (ISGF-3), a complex consisting of Stat1, Stat2 and p48 (Schindler *et al.*, 1992a,b; Veals *et al.*, 1992). In contrast, the activation of genes with GAS enhancers is mediated by the activation of Stat1 homodimers (Lew *et al.*, 1991; Khan *et al.*, 1993; Pine *et al.*, 1994). Similarly, IL-3 activates two different STFs: STF−IL-3a is activated in immature myeloid cells and STF−IL-3b is activated in more mature myeloid cells.

In an effort to study signal transduction by the IL-3 family of ligands, STF−IL-3a was purified and its component 77 and 80 kDa proteins examined. Both amino acid and nucleotide sequence data demonstrated that p77 and p80 are closely related but are encoded for by distinct genes. Additionally, both genes bear a striking homology to ovine MGF/Stat5, encoding many of the conserved domains that have been identified for other STAT proteins (Fu *et al.*, 1992; Hou *et al.*, 1994; Zhong *et al.*, 1994). Furthermore, our data indicate that Y699 is phosphorylated in the activated (i.e. GAS binding) conformation of p77 and p80. This tyrosine corresponds to the one that has been shown to be phosphorylated in a ligand-dependent manner in Stat1 and Stat2 (Schindler *et al.*, 1992b; Shuai *et al.*, 1993; Improta *et al.*, 1994), consistent with the possibility that this is the activation site of Stat5 (Gouilleux *et al.*, 1994).

An examination of the full-length p80 cDNA has led to several notable observations. First, the high degree of similarity between the p77 and p80 cDNAs (data not shown) suggests that this pair arose from a gene duplication

event. Interestingly, in mice there is an analogous duplication of IL-3 receptor β-chain, leading to the speculation that these gene duplications may be causally related. However, this will require further investigation. Second, as discussed above (see Figure 3B), the amino acid homology with the C-terminus of MGF/Stat5 switches reading frames after amino acid 715. This frame shift is unlikely to be due to an alternative splicing event because of the nature of the homology. Furthermore, PCR amplification of murine genomic DNA has failed to detect an intron in this region (unpublished observation). Third, the p80 cDNA encodes for a protein that is apparently too large. This paradox is unlikely to be due to anomalous migration during gel electrophoresis, since the apparent molecular weights of all STATs characterized to date are larger than their predicted molecular weight. However, the notion of a pre- or post-translational processing event, which would explain how a cDNA predicted to encode a 90 kDa protein gives rise to an 80 kDa protein, is consistent with several observations. For example, p77/p80 homologous transcripts are expressed in mature myeloid cells, where a larger related set of proteins has been detected. Moreover, both the p77/p80 and p94/p96 sets of proteins appear to be tyrosine-phosphorylated in response to IL-3 in distinct tissues. Furthermore, the smaller protein in the p94/p96 doublet is more readily detected by abSt5, a pattern that is suspiciously similar to the higher affinity that this antibody has for p77 in the p77/p80 doublet. These observations are most consistent with the possibility that p94 and p96 represent the full-length products of the p77 and p80 clones, and that the p77 and p80 proteins represent shorter gene products of the same genes. The p77 and p80 peptide data also support the possibility that these proteins are processed. For example, the truncation of p80 at amino acid 714 (e.g. peptide 80T15, Table I) would result in a protein of 82 kDa, significantly closer to the apparent molecular weight of 80 kDa. Future efforts will be directed at examining the hypothesis that the two characterized Stat5 genes encode for four distinct gene products.

It should be recognized that the identification of multiple gene products from one STAT gene is not without precedent. The Stat1 gene gives rise to two alternatively spliced gene products, p84 (Stat1α) and p91 (Stat1β). The shorter gene product, p84, is missing the C-terminal 38 amino acids and is unable to mediate the activation of GAS-driven genes (Müller *et al.*, 1993). This observation, as well as the fact that the STAT sequences diverge at their C-terminus, suggest that the C-terminal region is important for transcriptional activation. This raises the possibility that the shorter p77 and p80 gene products may have a different transcriptional activity to the larger p94 and p96 gene products. Consistent with these observations, a GAS-driven reporter construct is much more weakly induced by IL-3 when transfected into immature myeloid cells than when transfected into mature myeloid cells (Rothman *et al.*, 1994). It will be of interest to determine whether the different functional responses to IL-3 (e.g. proliferation versus activation) can be attributed to the differential activation of these isoforms.

In summary, we provide evidence that the two members of the IL-3 family of ligands signal through the activation of Stat5. Studies in Mui *et al.* (1995) demonstrate that another member of this family of ligands, IL-5, also signals through Stat5. Our observation that there are two Stat5 genes, which appear to give rise to multiple isoforms of Stat5, provides for a potential differential regulation of Stat5 signaling. This regulation could then account for the ability of the IL-3 family of ligands to stimulate different responses in different target cells. The availability of cDNA probes and specific antisera will enable us to examine critically the role of these Stat5 isoforms in IL-3, IL-5 and GM-CSF signaling.

Materials and methods

Cell culture

Cell culture reagents were purchased from Gibco. U937, HepG2, DA-3(EpoR), 32Dcl(Epo1), WEHI, FDtrk, CTLL, BAC1.2f5, EL-4, RL-12, NSF-70 and NSF-112 were grown as described previously (Morgan *et al.*, 1987; Ihle and Askew, 1989; Katzav *et al.*, 1989; Rothman *et al.*, 1994). MOLT4 cells were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS). 3T3 and COS-7 cells were grown in DMEM supplemented with 10% FCS. Prior to treatment with cytokines, growth factor-dependent cells were starved of these factors for 16 h. Cytokine treatments were as follows. U937 cells were stimulated with 3 ng/ml IFN-γ (Hoffmann-La Roche). HepG2 cells were treated with 200 U/ml IL-6 (a generous gift from S.Chen-Kiang). DA-3(EpoR), 32Dcl(Epo) and FDtrk cells were treated with either 10 U/ml IL-3 or 10 U/ml Epo (Amgen). U937 and MOLT4 cells were stimulated with 50 U/ml GM-CSF (Schering-Plough).

DNA binding assays and cell extracts

The preparation and employment of DNA oligonucleotide probes for mobility shift assays have been described previously (Bonni *et al.*, 1993; Pine *et al.*, 1994). Cytoplasmic, nuclear or whole-cell extracts were made as described previously (Schindler *et al.*, 1992b; Eilers *et al.*, 1993; Pine *et al.*, 1994). STAT antibodies were added (final dilution 1:20) for 30–60 min at 4°C after a standard shift reaction (e.g. 20 min at 25°C), or they were incubated with extracts for 30–60 min at 4°C prior to a standard shift reaction. Oligonucleotide precipitation assays were carried out as described previously (Rothman *et al.*, 1994). All shift reactions were performed with the IRF1 GAS probe gatcGATTTCCCCGAAAT (Oligos Etc.).

Antibodies

Polyclonal rabbit antisera ab1 and ab4 were generated against domains of p91 expressed as GST fusion proteins, as described previously (Schindler *et al.*, 1992a). ab1 recognizes p91 amino acids 2–66; ab4 recognizes amino acids 515–607. abSt5 was generated against the 300 bp PCR product, encoding amino acids 613–711, by cloning it into the *Eco*RI and *Bam*HI sites of pGEX-2T (Pharmacia). This GST fusion protein was used to immunize rabbits as described previously (Schindler *et al.*, 1992a). For abSt5, IgG fractions were prepared from crude immune rabbit serum (Pierce ImmunoPure IgG kit). Antiphosphotyrosine antibodies were purchased from UBI (4G10).

Purification and cloning

Two sets of whole-cell extract (without detergent; Eilers *et al.*, 1993) were prepared, each from 15 l of IL-3-stimulated FDtrk cells. They were sequentially fractionated on a heparin agarose (50 ml; Sigma) and a GAS trimeric oligonucleotide affinity column (0.5 ml; Rothman *et al.*, 1994). After washing with two column volumes, STF−IL-3a was eluted from the heparin agarose with a linear NaCl gradient (0.15–1.50 M in buffer C; Dignam *et al.*, 1983). STF−IL-3a was loaded onto an oligonucleotide matrix in low salt (150 mM NaCl) and recovered with a 1 M NaCl batch elution (in Buffer D; Rothman *et al.*, 1994).

Degenerate nucleotides based on the peptide sequences VFEPVVQ [CGGGATCCGG(T/G)ACCTTC(C/T)T(G/A)(C/T)T(G/T)(A/C)G(A/G/C/T)] and GTFLLRF [GGAATTCAC(A/G)AA(C/T)TCAGG(A/G/C/T)AC(A/G/C/T)AC(T/G)TG] (Oligos Etc.) were used to amplify a 300 bp product from a murine thymocyte cDNA library (Stratagene). Conditions for the PCR were 1 μl of phage library, 40 pmol of primers, denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min. This 300 bp PCR product was radiolabeled (Amersham dCTP at 3000 Ci/mmol) by random priming (Boehringer Mannheim).

Both the murine thymocyte phage library (1×10^6 plaques) and a murine bone marrow-derived mast cell (Domen *et al.*, 1993) pcDNA-3 library (Invitrogen) were screened by standard protocols. cDNA clones were sequenced on an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, Taq DyeDeoxy sequencing kit). RNA was prepared from tissues and cells and then Northern blotted by standard protocols (Beckers *et al.*, 1994).

Protein analysis

Affinity-purified proteins were precipitated and fractionated by SDS−PAGE, electroblotted onto a nitrocellulose membrane and visualized by Ponceau S staining. Bands of interest (p77 and p80) were excised and subjected to *in situ* proteolytic cleavage with 1 μg trypsin (Promega) in 25 μl 100 mM NH_4HCO_3, 10% acetonitrile and 0.3% Tween-80 at 37°C for 3 h, as described previously (Tempst *et al.*, 1990; Erdjument-Bromage *et al.*, 1994). The resulting peptide mixture was fractionated by reversed-phase HPLC as described (Elicone *et al.*, 1994), except that a 2.1 mm Vydac C4 (214TP54) column was used with gradient elution at a flow rate of 100 μl/min. The identification of Trp-containing peptides was performed by manual ratio analysis of absorbencies at 297 and 277 nm using a diode array detector (Erdjument-Bromage *et al.*, 1994).

Selected peptides were analyzed by a combination of automated Edman degradation and matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (Elicone *et al.*, 1994; Geromanos *et al.*, 1994). Mass analysis (on 2% aliquots) was carried out using a model LaserTec Research MALDI-TOF instrument (Vestec) and α-cyano-4-hydroxy cinnamic acid (Linear Sci.) as the matrix. Chemical sequencing (on 95% of the sample) was performed using a model 477A instrument (Applied Biosystems) with 'on-line' analysis (120A HPLC system with 2.1×220.0 mm PTH C18 column; Applied Biosystems), as described (Tempst *et al.*, 1994).

Peptide sequences were compared with entries in various sequence databases using the National Center for Biotechnology Information (NCBI) BLAST program (Altschul *et al.*, 1990). Average isotopic masses of the peptides were summed from the identified amino acids using ProComp version 1.2 software (obtained from Dr P.C.Andrews, University of Michigan, Ann Arbor, MI).

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Note added in proof

We have recently demonstrated that C-terminal antisera fail to recognize p77 and p80, consistent with our model of C-terminal processing. Also, a corrected version of the MGF sequence has recently been deposited in the EMBL data library under accession number X7828.