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The localisation of the heparin binding sites of human and murine interleukin-12 within the carboxyterminal domain of the P40 subunit

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ABSTRACT

We have previously shown that the heterodimeric cytokine interleukin-12, and the homodimer of its larger subunit p40, both bind to heparin and heparan sulfate with relatively high affinity. In the present study we characterised these interactions using a series of chemically modified heparins as competitive inhibitors. Human interleukin-12 and p40 homodimer show indistinguishable binding profiles with a panel of heparin derivatives, but that of murine interleukin-12 is distinct. Heparin markedly protects the human and murine p40 subunits, but not the p35 subunits, from cleavage by the bacterial endoprotease LysC, further implicating the larger subunit as the location of the heparin binding site. Moreover the essential role of the carboxyterminal D3 domain in heparin binding is established by the failure of a truncated construct of the p40 subunit lacking this domain to bind. Predictive docking calculations indicate that a cluster of basic residues at the tip of the exposed C′D′ loop within D3 is important in heparin binding. However since the human and murine C′D′ loops differ considerably in length, the mode and three dimensional orientation of heparin binding are likely to differ substantially between the human and murine p40s. Thus overall the binding of IL-12 via its p40 subunit to heparin-related polysaccharides of the extracellular matrix appears to be functionally important since it has been conserved across mammalian species despite this structural divergence.

1. Introduction

Interleukin 12 (IL-12) plays a key role in establishing cell-mediated, T_H1-type, immune responses by stimulating NK and T lymphocytes to secrete γ-IFN (for reviews see [1,2]). The major cellular sources of IL-12 are activated macrophages, monocytes and neutrophils. In turn, γ-IFN stimulates further production of IL-12, thus giving rise to a positive feedback loop at the initiation of T_H1 responses. IL-12 is a relatively large cytokine, M_r 70kD, being a heterodimer of two disulphide-bridged subunits, p40 and p35. The p40 subunit also occurs as a component of IL-23, in which the smaller p35 subunit is replaced by a structurally related subunit, p19 [2]. IL-23 is a pro-inflammatory and immunostimulatory cytokine expressed by antigen presenting cells upon activation. However IL-23 stimulates the T_H17 lymphocyte subset, thereby promoting both early responses to microbial infection and chronic autoimmune states. The p35 subunit is also found in IL-35, an immunosuppressive cytokine [3]. The high affinity cell surface signalling receptor for IL-12 is a heterodimer of the IL-12Rβ1 polypeptide, which is also a component of the IL-23 receptor, and IL-12Rβ2, which is shared with the IL-35 receptor [3]. Free p40 is also found in serum in a number of disease states and in efforts to identify the biological role of this polypeptide a number of binding proteins have been identified, including the CD5 glycoprotein [4].

The pivotal role of IL-12 in initiating and promoting T_H1 immune responses was convincingly demonstrated in IL-12 p40 deficient mice, which show greatly impaired γ-IFN responses to antigenic challenge [5], and are unable to mount an effective T_H1 response to *Mycobacterium tuberculosis* infection [6]. Moreover both $p40^{-/-}$ and $p35^{-/-}$

Abbreviations: BMP, bone morphogenetic protein; CS, chondroitin sulphate; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAG, glycosaminoglycan; GDNF, glial cell line-derived neurotrophic factor; γ-IFN, γ-interferon; HS, heparan sulfate; h, human; IL, interleukin; m, murine; NK, natural killer; r, recombinant; TNF, tumour necrosis factor

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mice, despite having an otherwise resistant genetic background, show susceptibility to *Leishmania major* infection and polarised T_H2 immune responses [7]. In humans, deficiencies in IL-12 production and signalling have been found to cause susceptibility to disseminated *Salmonella* and mycobacterial infections, including tuberculosis [8,9]. Based on previous evidence from experimental rodent models of disease, IL-12 and p40 have become targets of interest in a number of chronic human diseases with autoimmune and inflammatory aspects, including psoriasis, psoriatic arthritis, Crohn's disease, ulcerative colitis, rheumatoid arthritis and multiple sclerosis [reviewed in 10]. Ustekinumab, a humanised p40-specific monoclonal antibody which blocks IL-12 and IL-23 signalling [11], is now an approved therapeutic agent for psoriasis and psoriatic arthritis [12,13], and also for the treatment of moderate to severe Crohn's disease in patients refractory to anti-TNF treatment [14]. In addition IL-12 has long been recognised to have anti-tumour activities [15], and the localised delivery of IL-12 remains under investigation in the experimental immunotherapy of cancer [16–18].

The p35 subunit of IL-12 shares weak sequence homology with IL-6, a 4 α-helix bundle cytokine [19]. Likewise an extensive if weak sequence homology has been noted between p40 and the IL-6 receptor polypeptide [20]. IL-12 can therefore be conceptualised as a 4-α helix bundle cytokine covalently pre-bound to a soluble class I cytokine receptor chain. The high resolution crystallographic structures of human IL-12 accord entirely with this paradigm [21,22]. The p35 subunit indeed has a 4 α-helix conformation, whereas p40 has 3 distinct fibronectin-like Ig domains. The p35 subunit docks into a hinge region between the central p40 domain, D2, and the carboxyterminal p40 domain, D3. The epitope for ustekinumab lies within the aminoterminal domain of p40, D1 [22]. Recent structural studies of IL-23 show that briakinumab, another IL-12 and IL-23 blocking monoclonal antibody, binds to p40 at the same site [23].

We have previously reported that human and murine IL-12 bind with comparatively high affinity to the sulfated glycosaminoglycans (GAGs) heparin and heparan sulfate, HS [24]. This binding appears to involve highly sulfated motifs within the GAG chains. Since several 4 α-helix cytokines including ILs-2 [25], -4 [26], -5 [27], -6 [28], and -7 [29], are heparin-binding cytokines, it might be surmised that IL-12 binds to sulfated GAGs via p35. However our finding that the human p40 dimer binds to heparin and HS in a manner indistinguishable from human IL-12 indicates instead that the binding site resides within p40 [24].

The binding of a cytokine to heparin-related polysaccharides of the extracellular matrix and cell surface will serve to retain the cytokine close to its site of secretion within tissue microcompartments [30]. Given the biological role of IL-12 as an activating signal for T and NK lymphocytes secreted by antigen presenting cells, the localised delivery of IL-12 is likely to be physiologically significant in the context of the immunological synapse which forms as a tight physical contact between antigen presenting cells and T cells. Interestingly γ-IFN, which is produced in response to IL-12, is also a heparin/HS binding cytokine [31], suggesting that these GAGs are important in localising the initiation of T_H1 immune responses.

As has been well studied in the case of FGFs, heparin/HS may also function as cytokine co-receptors, by participating in the formation of cytokine-receptor complexes and thereby promoting signalling activity [reviewed in 32]. Whether sulphated GAGs function as co-receptors for IL-12 remains unclear, although we have shown that stripping the murine natural killer cell line KY-1 of the GAG chondroitin sulfate B, greatly reduces responsiveness to IL-12 in terms of interferon-γ secretion, and that chondroitin sulfate B competes for the heparin binding site on IL-12, albeit with lower affinity [33].

Here we investigate further the structural basis of the interaction between IL-12 and sulphated GAGs. We report experimental findings which clearly implicate the carboxyterminal domain, D3, of the p40 subunit as the site of heparin binding. We have also employed theoretical docking calculations which predict that key amino acid residues involved in heparin binding are located in basically charged clusters on flexible, exposed polypeptide loops near the carboxyterminus of p40 D3, a region of appreciable structural divergence between murine and human IL-12.

2. Materials and methods

2.1. Materials

Recombinant murine and human IL-12s (rmIL-12 and rhIL-12), rhIL-12 p40, and goat polyclonal anti-rmIL-12 and anti-rhIL-12 were purchased from R&D Systems Europe Ltd. (Abingdon, Oxon, UK). Alkaline phosphatase- and horseradish peroxidase-coupled anti-goat Ig, endoproteinase LysC (EC 3.4.21.50) and *p*-nitrophenyl phosphate tablets were all obtained from the Sigma Chemical Co., St. Louis, MO, USA. A series of chemically modified heparins, were prepared and structurally characterised as fully described elsewhere [34]. PCR primers were obtained from MWG Biotech (Edersberg, Germany). Murine IL-12 p35 and p40 cDNA clones in pBluescript were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Restriction endonucleases were purchased from Promega Corporation (Madison, WI, USA).

2.2. Heparin binding ELISA, proteolytic digestion and Western blotting

The binding of rIL-12 and its derivatives to an immobilised heparin-BSA complex was studied by ELISA as described previously [24,25]. For digestion with endoproteinase LysC, rIL-12 and p40 (1.25 μg/ml) were incubated with 2 μg/ml enzyme in 20 mM Tris–HCl buffer, pH8.0, at 37 °C. Aliquots, 20 μl, were removed at different digestion times and immediately boiled in SDS sample buffer. Digested samples were separated by SDS PAGE on 15% (wt/vol) polyacrylamide gels and electroblotted on to nitrocellulose. All other samples for Western blotting were separated on 12% (wt/vol) gels. After blocking in PBS containing 5% (wt/vol) fat-depleted dried skimmed milk powder and 0.05% (vol/vol) Tween for 30 min, followed by washing in PBS, the blot was incubated overnight at 4 °C in blocking buffer containing goat anti-IL-12 antibody at 1/2000 dilution. After washing in blocking buffer, the blot was incubated with peroxidase-coupled anti-goat Ig diluted 1/2000 for 45 min. Finally IL-12 immunoreactivity was detected by chemiluminescence reagents (SuperSignal West Pico, Perbio, Tattenhall, Cheshire, UK).

2.3. Murine IL-12 gene constructs and site directed mutagenesis

Murine p35 and p40 cDNAs were cloned directly into the pTriEx vector (Novagen, Darmstadt, Germany) for expression in mammalian cells. Following the approach of Lieschke et al. [35], a single chain construct, psc70, was generated by splicing the sequence for p40 at its carboxyterminus via a flexible linker, $(Gly_4Ser)_3$, to codon 23 of the p35 cDNA, which encodes Arg, the first residue following the presumptive signal sequence. Within the p40 cDNA, an *Nco1* restriction site was introduced at the aminoterminal end by a single base change in the second codon (TGT to GGT), and the stop codon was deleted at the 3′ extremity. These changes were introduced by PCR amplification using the following primers: p40-*Nco1* (sense), 5′-CAAAGCACCTAGGGTCCT CAGAAG-3′; p40-*Nco1* (antisense), 5′-TTTAAAGGATCCACCACCGCCC GAGCCACCGCCACCGGATCGGACCCT-3′; p35-linker (sense), 5′-AAAT TTGGATCCGGTGGCGCCGGATCTAGGGTCATTCCAGTCTCTGGA-3′; p35-C terminus (antisense) 5′-AAAAGCTGGAGCTCCACCGCG-3′, with the *Nco1* restriction sites shown underlined. P40 and p35 PCR products were respectively digested with *Nco1/BamH1* and *BamH1/Not1* enzymes, purified electrophoretically, ligated and subcloned into the pGEM-T Easy vector (Promega, Southampton, Hants., UK).

In order to produce the p40 variant, p40ΔD3, which lacks the D3 domain, a stop codon was introduced at the end of the D2 codon by PCR