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Targeting cytokines to inflammation sites

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To increase the half-life of a cytokine and target its activation specifically to disease sites, we have engineered a latent cytokine using the latency-associated protein (LAP) of transforming growth factor-β1 (TGF-β1) fused via a matrix metalloproteinase (MMP) cleavage site to interferon (IFN)-β at either its N or C terminus. The configuration LAP-MMP-IFN-β resembles native TGF-β and lacks biological activity until cleaved by MMPs, whereas the configuration IFN-β-MMP-LAP is active. LAP provides for a disulfide-linked shell hindering interaction of the cytokine with its cellular receptors, conferring a very long half-life of 55 h *in vivo*. Mutations of the disulfide bonds in LAP abolish this latency. Samples of cerebrospinal fluid (CSF) or synovial fluid from patients with inflammatory diseases specifically activate the latent cytokine, whereas serum samples do not. Intramuscular injection in arthritic mice of plasmid DNA encoding these constructs demonstrated a greater therapeutic effect of the latent as compared to the active forms.

Cytokine gene expression is generally controlled at the transcriptional and post-transcriptional levels[1,2]. Another level of regulation is found in cytokines that interact with the extracellular matrix (ECM)[3], such as TGF-β, which is secreted in a latent form, binds to the ECM and becomes 'activated' by releasing the cytokine moiety at sites where processes of inflammation, wound healing and tissue repair take place[4].

Cytokines are natural products that serve as soluble local mediators of cell-cell interactions. They have short half-lives and a variety of pleiotropic actions, some of which can be harnessed for therapeutic purposes[5,6]. Unfortunately, these very potent biological agents have to be administered at very high concentrations systemically to achieve biologically meaningful concentrations in the tissue being targeted. This gives rise to toxic systemic effects that limit their use and efficacy[7–9].

To bypass the toxic effects of systemic administration, we have engineered a cytokine that uses the LAP of TGF-β1 as a protective 'shell' preventing it from interacting with its receptors. Our engineered latent cytokine also possesses an MMP cleavage site that is cut at sites of inflammation and tissue remodeling, releasing the active cytokine and enabling it to act on cells locally. To our knowledge, this is the first description of a fusion protein enabling action of a cytokine at sites of inflammation. Other cytokine fusion proteins, such as immunocytokines, target cytokines using antibodies to specific cell types expressing defined antigens[10]; previous fusion proteins of TGF-β[11,12] have focused on the active cytokine moiety but not its LAP.

RESULTS

Structural considerations

To develop a latent cytokine using the LAP domain of TGF-β, we built fusion proteins in two conformations, one containing LAP at the N terminus of mouse IFN-β and the other at the C terminus. Cysteines 224 and 226 are important in the intermolecular disulfide bond between two LAPs. Their mutation to serine renders the molecule 'active'[13–15]. The RGD motif (residues 245–247) facilitates the interaction with integrins[16,17]. Cysteine 33 is important for the disulfide bridge with the third eight-cysteine-rich repeat of latent TGF-β binding protein (LTBP)[18]. Modification of LTBP by other enzymes such as thrombospondin[19,20], transglutaminase[21,22], and MMP9 and MMP2 (ref. 23) could release the active, noncovalently bound portion of TGF-β from the latent complex.

To prevent processing of the LAP-IFN-β protein at Arg278 of LAP, we cloned LAP spanning amino acids Met1–Ser273. This sequence was followed by a flexible linker (GGGGS), a putative MMP9 (refs. 24,25) or putative MMP1 (ref. 26) cleavage site (PLGLWA), and another flexible portion (GGGGSAAA) followed by mature IFN-β (starting at Ile22). We expected that embedding the MMP cleavage site in a hydrophilic area would facilitate access to enzymatic attack. The core of the cleavage site (PLGL) is cleaved as a peptide by MMP2 and, in a different version (PLGI), also by MMP3, MMP7 and MMP8 (ref. 26).

The IFN-β-LAP molecule consisted of the precursor IFN-β sequence, with its stop codon mutated to allow readthrough of the flexible linker and the MMP site, followed by the mature sequence of LAP (Leu29–Ser273). The unprocessed LAP-IFN-β and IFN-β-LAP fusion proteins have expected molecular weights of 52.375 and 51.768 kDa, respectively. The primary sequences of these fusion proteins each contain four possible *N*-glycosylation sites. A schematic representation of the primary structure and putative folding of these proteins and their interaction with LTBP are shown in **Figure 1a,b**.

We expressed these recombinant proteins in dihydrofolate reductase–deficient Chinese hamster ovary (DHFR$^-$ CHO) cells using a permanent DNA-transfection process. Secreted IFN-β-LAP had a low residual biological activity (210 U/ml), whereas LAP-IFN-β was completely 'latent' or inactive. The expression of these recombinant

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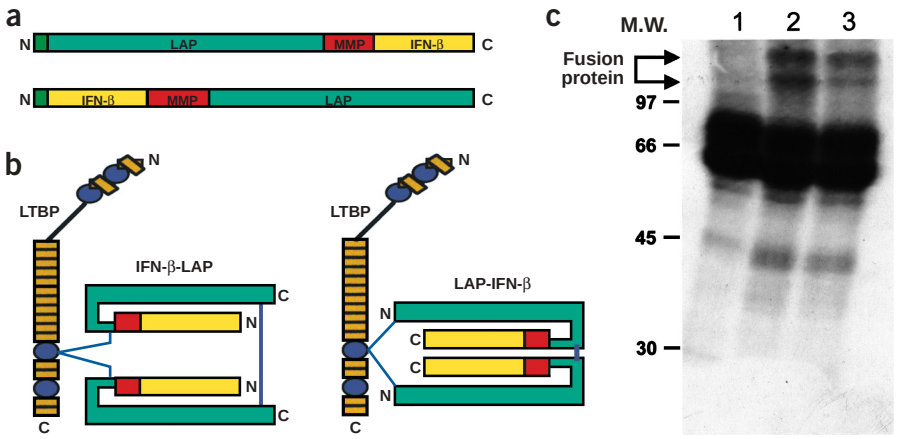

Figure 1 Schematic representation of fusion proteins and their putative folding. (**a**) Primary structure of recombinant latent proteins. The linear sequence arrangement of the constituents in the two configurations is shown. The green box at the N terminus depicts the native signal sequence peptide for secretion of either TGF-β or IFN-β, respectively. (**b**) Putative folding and interactions with LTBP of latent cytokine. Right, folding of LAP-IFN-β, resembling the folding of native TGF-β. Near the N terminus of the protein, Cys33 interacts with the third eight-cysteine-rich repeat of LTBP, whereas Cys224 and Cys226 dimerize the protein through intermolecular disulfide bonds[27]. Left, structure of IFN-β-LAP. Cys33 is now located behind the MMP cleavage site and Cys224 and Cys226 are closer to the C terminus of the protein. In LTBP, the epidermal growth factor–like repeats are shown as small, square yellow boxes, the cysteine-rich repeats and hybrid domain as blue circles, and the 'hinge region,' which is sensitive to proteolytic cleavage, as a line. Disulfide bonds are shown as blue lines. (**c**) Detection of recombinant fusion proteins in cell supernatants. Nondenaturing SDS-PAGE of supernatants from nontransfected (lane 1), LAP-IFN-β-transfected (lane 2) and IFN-β-LAP-transfected CHO cells (lane 3). Positions of the double bands of newly expressed fusion proteins are marked by a double arrow. Positions of the molecular weight markers (M.W.) in kilodaltons are shown.

proteins was similar, as confirmed by Western blotting with an anti-LAP antibody (data not shown).

Biochemical characterization of recombinant proteins

Secreted proteins from permanently transfected CHO cells were labeled with [^{35}S]methionine and [^{35}S]cysteine. When subjected to electrophoresis under nonreducing conditions, the labeled LAP-IFN-β and IFN-β-LAP proteins each produced two major bands of more than 97 kDa that were not seen in supernatants from nontransfected CHO cells (**Fig. 1c**). Upon immunoprecipitation with anti-LAP antibody in reducing conditions, supernatants from LAP-IFN-β- and IFN-β-LAP-transfected cells showed three bands, one of 57 kDa and another of 135 kDa and a minor component at around 75 kDa (**Fig. 2a**). The 135-kDa protein is probably the CHO-derived LTBP, which is linked by disulfide bonds to LAP[27].

Table 1 IFN-β activity of supernatants of MTX-amplified, fusion protein–transfected CHO cells

	IFN-β biological activity (U/ml) after treatment with:							
Cytokine construct	No MMP	MMP1	MMP3	Pro-MMP9	Pro-MMP9 + MMP1	Pro-MMP9 + MMP3	RASF	No SPI
LAP-IFN-β (50 nM MTX)	288	6,144	9,216	288	1,536	768	1,152	768
IFN-β-LAP (12.5 nM MTX)	1,536	6,144	3,072	1,536	1,536	4,608	6,144	3,072

Supernatants were supplemented with or without (last column) serine protease inhibitors (SPI) and MMPs as indicated. Incubations were overnight at 37 °C and tested for IFN-β activity. Supernatants from nontransfected CHO cells had no IFN-β activity even after treatment with MMPs or synovial fluid from patients with rheumatoid arthritis (RASF) at 1/5 of final volume (data not shown). The RASF is the same as used in **Figure 3**.

After gene amplification with methotrexate (MTX), the minor 75-kDa component (**Fig. 2a**, lanes 1, 3, 5) became the major component recognized by anti-LAP antibody (**Fig. 2b**, lanes 1–4). The approximately six- to eightfold increase of the fusion protein over LTBP indicates that interaction with LTBP is not a requirement for latency of LAP-IFN-β (see basal levels of activity in **Fig. 3** and **Table 1**). Interestingly, the monoclonal anti-IFN-β antibody does not seem to recognize the 75-kDa glycosylated product (**Fig. 2b,c**, lanes 5–8) and the anti-LAP antibody recognizes it poorly in the IFN-β-LAP configuration (**Fig. 2b**, lanes 5–8), indicating that these fusion proteins have different conformations. This may explain the different sensitivities of these proteins to various MMPs (see below) and their differing degree of latency. The calculated molecular weight of the secreted recombinant proteins is 49.376 kDa for both LAP-IFN-β and IFN-β-LAP. The higher molecular weight is due to glycosylation of these proteins. Incubation with *N*-glycosidase F yields two major proteins of molecular weights 70 kDa and 51 kDa, corresponding to LTBP and fusion protein, respectively (data not shown).

MMP cleavage of recombinant proteins

Immunoprecipitated complexes were treated overnight with MMPs either singly or in combination. MMP1 did not cleave the 57-kDa recombinant product very efficiently (**Fig. 2a**). MMP1 cleaved the glycosylated form of the fusion protein (**Fig. 2a**, lanes 5, 6 and **Fig. 2b**, lane 2), whereas MMP3 digested it into several discrete bands (**Fig. 2a**, lanes 3, 4 and **Fig. 2b**, lanes 3, 7). The LTBP band was also cleaved by MMP3 (**Fig. 2a**, lanes 3, 4 and **Fig. 2c**, lanes 3, 7), giving rise to a 78-kDa product. Two of the digested products (43 kDa and 32 kDa) corresponded to the expected LAP and IFN-β polypeptide fragments, respectively, as assessed by western blotting with the respective antibodies (data not shown).

The specificity shown in these *in vitro* experiments does not fully reflect the antiviral activity measured in cell supernatants after MMP treatment. Cell supernatants were already activated to a certain extent, indicating that other proteinases in the supernatant may activate the latent cytokine moiety. We did not see increased proteolysis of the fusion polypeptides after immunoprecipitation using a combination of recombinant pro-MMP9 with MMP1 or MMP3, or with aminophenylmercuric acetate (APMA)-activated pro-MMP9 on its own *in vitro* (data not shown), suggesting that MMP9 does not cleave the fusion proteins directly.

Cell supernatants were concentrated 100-fold by centrifugation through porous membranes to allow for MMP activity at a higher substrate concentration. LAP-IFN-β supernatant showed antiviral activity without any further treatment (163 U/ml). This is not surprising, as CHO cells are reported to secrete a variety of proteinases[28,29], including MMPs[30].

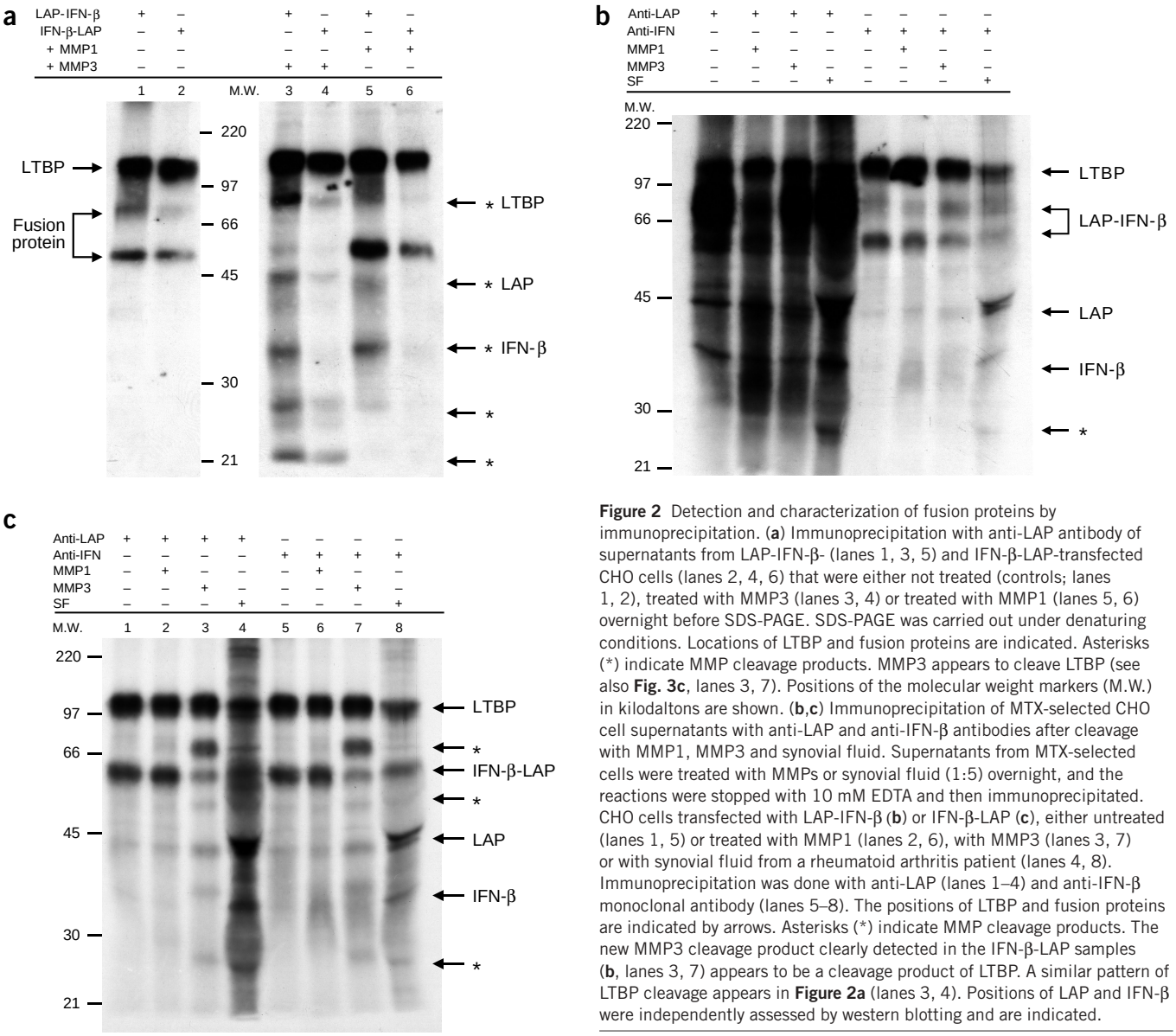

Figure 2 Detection and characterization of fusion proteins by immunoprecipitation. (**a**) Immunoprecipitation with anti-LAP antibody of supernatants from LAP-IFN-β- (lanes 1, 3, 5) and IFN-β-LAP-transfected CHO cells (lanes 2, 4, 6) that were either not treated (controls; lanes 1, 2), treated with MMP3 (lanes 3, 4) or treated with MMP1 (lanes 5, 6) overnight before SDS-PAGE. SDS-PAGE was carried out under denaturing conditions. Locations of LTBP and fusion proteins are indicated. Asterisks (*) indicate MMP cleavage products. MMP3 appears to cleave LTBP (see also **Fig. 3c**, lanes 3, 7). Positions of the molecular weight markers (M.W.) in kilodaltons are shown. (**b**,**c**) Immunoprecipitation of MTX-selected CHO cell supernatants with anti-LAP and anti-IFN-β antibodies after cleavage with MMP1, MMP3 and synovial fluid. Supernatants from MTX-selected cells were treated with MMPs or synovial fluid (1:5) overnight, and the reactions were stopped with 10 mM EDTA and then immunoprecipitated. CHO cells transfected with LAP-IFN-β (**b**) or IFN-β-LAP (**c**), either untreated (lanes 1, 5) or treated with MMP1 (lanes 2, 6), with MMP3 (lanes 3, 7) or with synovial fluid from a rheumatoid arthritis patient (lanes 4, 8). Immunoprecipitation was done with anti-LAP (lanes 1–4) and anti-IFN-β monoclonal antibody (lanes 5–8). The positions of LTBP and fusion proteins are indicated by arrows. Asterisks (*) indicate MMP cleavage products. The new MMP3 cleavage product clearly detected in the IFN-β-LAP samples (**b**, lanes 3, 7) appears to be a cleavage product of LTBP. A similar pattern of LTBP cleavage appears in **Figure 2a** (lanes 3, 4). Positions of LAP and IFN-β were independently assessed by western blotting and are indicated.

Possibly the concentration step removed some natural inhibitors of MMPs (tissue inhibitor of metalloproteinase), facilitating their activity.

Addition of MMP1 to concentrated supernatants slightly increased the biological activity, as did addition of both MMP1 and pro-MMP9 or MMP3 and pro-MMP9 (data not shown). Interestingly, treatment of IFN-β-LAP with MMP1 and pro-MMP9 led to three- and sixfold increases in antiviral activity, respectively (from 1,300 U/ml to 3,480 and 7,740 U/ml), indicating that further activation of this molecule is possible.

Using nonconcentrated supernatants from MTX-amplified cells, we showed that both MMP1 and MMP3 activated LAP-IFN-β by 21- and 32-fold, respectively, and that synovial fluid from rheumatoid arthritis patients activated it by up to 4-fold (**Table 1**). Synovial fluid from patients with rheumatoid arthritis also cleaved the fusion proteins into discrete products of expected size (**Fig. 2b**,**c**, lanes 4 and 8). The sensitivity of the two fusion proteins to the presence of MMP9 is different: IFN-β-LAP can be activated, whereas LAP-IFN-β appears to be inhibited, perhaps inducing its further degradation by other enzymes present in the CHO cell supernatants.

Disulfide bonds are required for latency

To assess whether the latency detected with LAP-IFN-β required the formation of a putative closed 'shell' structure bounded by the double disulfide–linked LAP, we constructed a fusion protein using the porcine LAP in which Cys223 and Cys225 were mutated to serines. We compared this PorcLAP-IFN-β to the other constructs with respect to its biological activity *in vitro* after transient transfection into COS-7 cells. PorcLAP-IFN-β was as active as IFN-β-LAP in this assay (256 U/ml) (representative result of two independent experiments done in duplicate) whereas LAP-IFN-β did not show any biological activity. This result demonstrated that the 'shell' structure requires the double disulfide bond of LAP.

Activation of latent IFN-β with fluids from inflamed sites

To determine whether long-term incubation of the fusion proteins

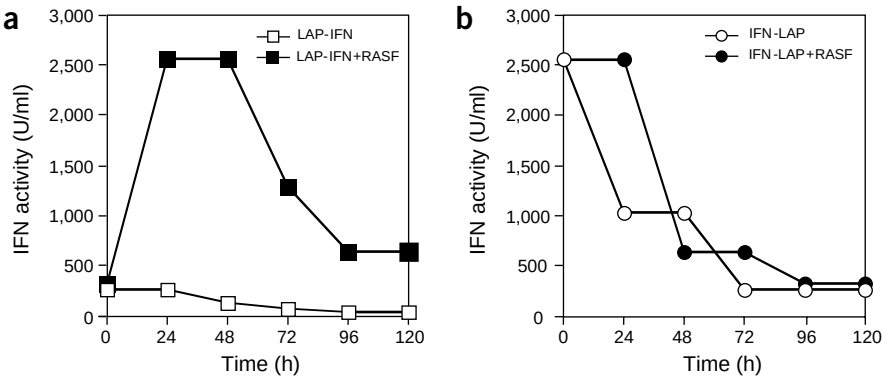

Figure 3 Kinetics of IFN activity after incubation of cell supernatants in medium alone or with synovial fluid from rheumatoid arthritis patients. (**a**) LAP-IFN-β-transfected cells. (**b**) IFN-β-LAP-transfected cells. Samples were incubated for up to 5 d at 37 °C and then applied to the IFN assay. Open symbols indicate samples incubated in medium with 10% FBS, and filled symbols indicate samples incubated with 1:5 (vol/vol) rheumatoid arthritis patient–derived synovial fluid (RASF). The RASF is different than the one used in **Table 1** or **Figure 2b**,**c**. Data is representative of two independent experiments.

would lead to their degradation or accumulation into active compound, we incubated both LAP-IFN-β and IFN-β-LAP for 5 d in the presence or absence of synovial fluid from a rheumatoid arthritis patient and took samples at 24-h intervals. Incubation resulted in up to tenfold increased activity of the LAP-IFN-β during the first 24–48 h, followed by a steady decrease afterward (**Fig. 3**). The IFN-β-LAP was not activated; only a decrease in its activity was seen. These data indicate the conformation LAP-IFN-β is protected from degradation during its incubation in serum-containing medium and can be activated by synovial fluid of rheumatoid arthritis patients, and that in contrast, the IFN-β-LAP conformation is very sensitive to degradation in both.

To assess whether samples from patients with other pathological inflammatory conditions could activate LAP-IFN-β, we tested, using a blind study design, synovial fluid or CSF and paired sera from patients with osteoarthritis or neurological diseases. Serum samples did not activate the latent cytokine above background. Thus, fluid samples causing more than fourfold greater IFN biological activity than the paired serum were considered positive. After our collaborators disclosed the origins of the samples (**Table 2**), we determined that three of eight human CSF samples tested were positive, and these correlated with four samples from patients with oligoclonal antibodies in the CSF. Of these samples, three were from patients with multiple sclerosis and one from a patient with meningitis. We were unable to detect activity in one of the multiple sclerosis samples. Four CSF samples were negative and all were from patients with noninflammatory neurological conditions. Two of three synovial fluid samples from osteoarthritis patients were positive in this assay. The sensitivity of the activation *in vitro* could have varied because of differing storage conditions as well as the extent of disease activity at the time of sampling.

In addition, three parallel samples of serum and CSF from rhesus monkeys with experimental autoimmune encephalomyelitis (EAE) or collagen-induced arthritis (CIA) were tested for activation of LAP-IFN-β. CSF from monkeys with CIA did not activate the latent molecule, whereas CSF from monkeys with EAE did in all samples. Neither EAE nor CSF serum samples activated LAP-IFN above background.

Affinity purification of LAP-IFN-β and pharmacokinetic studies

Attempts to purify the fusion protein by conventional chromatographic procedures failed. Hence, the protein was tagged with a polyhistidine tail and expressed in inclusion bodies in *Escherichia coli*. The denatured protein was purified on nickel chelate columns (data not shown). However, its resuspension and folding were unsuccessful. Finally, serum-free supernatants from CHO cells expressing LAP-IFN-β and amplified in 500 nM MTX were supplemented with EDTA to avoid degradation by endogenous MMPs; the fusion protein was purified by affinity chromatography on goat anti–human LAP antibodies crosslinked to a solid support (**Fig. 4a**). The eluted single peak contained 98% pure LAP-IFN-β as assessed by western blotting (**Fig. 4b**) and iodination (**Fig. 4c**). The iodinated protein was then injected intraperitoneally into Wistar rats, and blood samples were drawn at the specified times (**Fig. 4d**). The average half-life of the protein in blood was 55.02 h. The distribution of radioactivity in tissues, as compared to the values in blood, were lower in heart (0.44-fold) and lungs (0.76-fold), and higher in liver, spleen (1.3-fold) and kidneys (4-fold). These data suggest that the protein is secreted mainly via the urine.

Gene delivery of LAP-IFN-β is the most efficient treatment of CIA

We have previously shown that IFN-β gene delivery has therapeutic effects in models of multiple sclerosis[31] and rheumatoid arthritis[32]. Thus, we assessed whether *in vitro* data obtained with our fusion proteins could be verified and further substantiated *in vivo*. We determined that our plasmid constructs could affect established arthritis when delivered by a single intramuscular injection of DNA. The control plasmid without insert (pcDNA3) has no effect on arthritis development as assessed from paw swelling (**Fig. 5a**) or clinical score (**Fig. 5b**). On the other hand, both the active IFN-β-LAP and PorcLAP-IFN-β inhibited established disease. More importantly, the latent construct, LAP-IFN-β, was more potent as a therapeutic agent in inhibiting paw swelling and reducing clinical score ($P < 0.05$ from day 8 onward as compared to pcDNA3). Similar results were obtained in an independent experiment using 10 μg DNA (data not shown).

DISCUSSION

We have shown that an active cytokine molecule could be designed to become 'latent' by addition of the latency domain of TGF-β at either its N or C terminus. The LAP domain of TGF-β conferred 'latency' to IFN-β, which could be abrogated by incubating the fusion protein with recombinant MMPs. The latency is a result of steric hindrance inhibiting the interaction between IFN-β and its cellular receptors. Despite the fact that both the N and C termini of the molecule are in

Table 2 Activation of LAP-IFN-β with fluids from patients with inflammatory diseases

Sample (fluid type)	Number of positive samples/total samples
Multiple sclerosis (CSF)	2/3
Meningitis (CSF)	1/1
Noninflammatory CNS disease (CSF)	0/4
Osteoarthritis (SF)	2/3

Paired samples of serum, cerebrospinal fluid (CSF; 1:5, vol/vol) or synovial fluid (SF) were incubated with LAP-IFN-β overnight at 37 °C and assayed on LTK$^-$ cells for IFN-β activity. Increased activity of CSF or SF over paired serum (greater than fourfold) was considered positive.

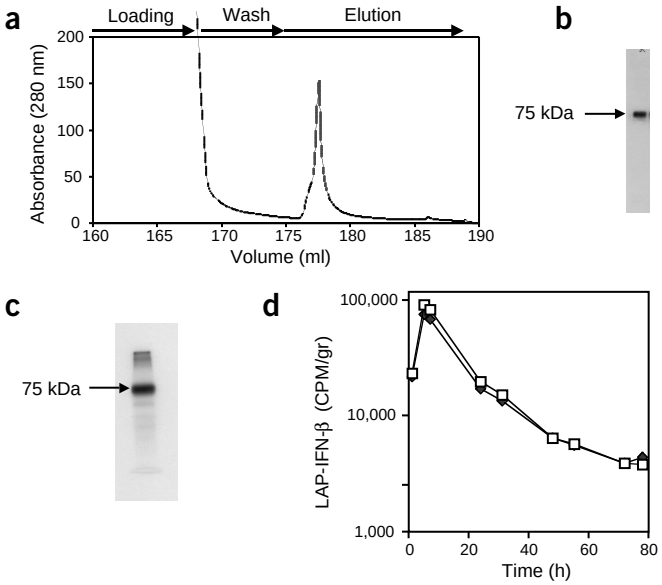

Figure 4 Affinity chromatography of LAP-IFN-β and pharmacokinetics. (**a**) 280 nm absorbance profile of fractions collected; arrows indicate when loading, washing and elution were done. (**b**) Western blot of peak fraction with anti-IFN-β antibody. (**c**) Autoradiograph of ^{125}I-labeled LAP-IFN-β from peak fraction. (**d**) Pharmacokinetic data from two Wistar rats injected intraperitoneally with iodinated LAP-IFN-β. Plot of c.p.m. iodinated LAP-IFN-β/g blood (log scale) were measured from 1 h to 78 h.

close proximity in the crystal structure of IFN-β[33], it is clear that a better 'shell' is conferred by fusing the LAP domain at its N terminus, as it is found in native TGF-β. It is interesting to note that the 'shell' structure can encapsidate a substantially bigger IFN-β (32 kDa) than the native TGF-β (12.5 kDa).

The MMP site located between LAP and IFN-β could be cleaved *in vitro* by MMP-3 and MMP-1. This is not surprising, as these MMPs have homologous regions in their active site[34]. Other MMPs could also cleave this site as shown by the activation occurring in concentrated serum-free supernatants of CHO cells or fluids from inflamed sites. MMP9 could not cleave our fusion proteins. Using fluorogenic peptide substrates with the sequence PLGLWA-D-R, the value of the rate of hydrolysis (k_{cat}/K_M) of MMPs appears to follow the order MMP9 > MMP2 > MMP7 > MMP3 > MMP1 (ref. 26). This discrepancy in hydrolysis sensitivity between the peptide substrate and our engineered proteins may be related to their tertiary structures.

The 'latent' cytokine design has a longer half-life. LAP-containing TGF-β has a longer half-life than free TGF-β *in vivo*[35]. We showed here that LAP-IFN has a half-life of 55.02 h when injected intraperitoneally in rats. Thus, the half-life of LAP-IFN is about 37.6 times longer than the reported half-life of IFN-β alone (1.46 h) and 5.08 times longer than that of improved pegylated IFN-β (10.82 h) injected by the same route[36]. The increase in the half-life of IFN-β (37.6-fold) in the context of LAP-IFN is of the same order of magnitude (30- to 50-fold) as that reported for TGF-β when it was associated with LAP *in vitro* before injection[35]. Thus, because of this longer half-life, the cytokine could be administered systemically using lower dosages. Secondly, the cytokine will not be released to interact with cellular receptors unless inflammatory or tissue-remodeling processes involving MMP activity are taking place. Expression of MMPs is very tightly regulated[37]. MMP activity is found in osteoarthritis, rheumatoid arthritis[38–40] and other chronic diseases such as inflammatory bowel disease[41,42], multiple sclerosis[43,44], atherosclerosis[45] and cancer during its invasive phase[46]. Thus, therapeutic agents, engineered as LAP-IFN-β was, could be used to treat these conditions.

We found that, when delivered by gene therapy using intramuscular injection, the latent cytokine was more effective than the active counterparts in the treatment of established arthritis. Upon cleavage, the release of LAP could have antagonistic effects for TGF-β, as LAP can inhibit active TGF-β action *in vitro*[47]. We expect, however, that our LAP fusion protein will act at sites of inflammation where free radicals abound. Nitrosylation of LAP disables its capacity for binding to TGF-β[48]. Thus, it is unlikely that, in sites of inflammation, the released LAP will antagonize TGF-β function.

The applications of this targeting approach for biologically active compounds are broad. Moreover, additional modifications to the MMP cleavage site may provide additional tissue or disease specificity. Such engineered latent cytokines, having a longer half-life and increased specificity, will help in reducing the cost of cytokine treatment for patients, as they will be more efficient at lower concentrations than the free cytokines used today.

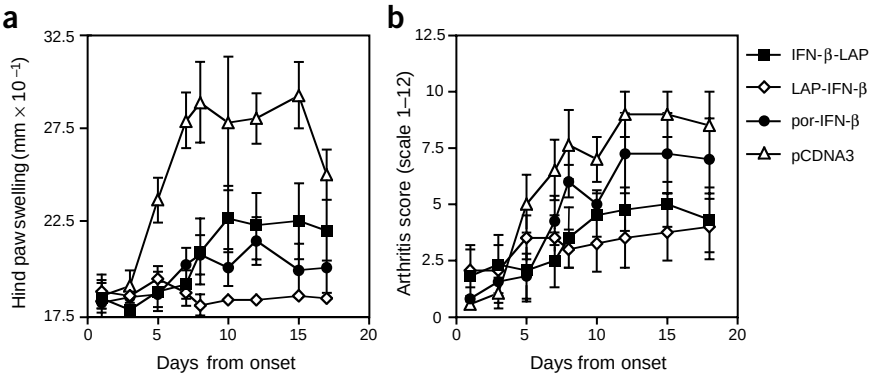

Figure 5 Inhibition of established collagen-induced arthritis by DNA injection with LAP-IFN-β. (**a**,**b**) Male DBA/1 mice (n = 4 per group) were immunized with CII and, at onset of disease (clinical score of 1), injected once intramuscularly with plasmid DNA as shown. Hind paw swelling (**a**) and arthritis score (**b**) were assessed as described in Methods. Data from one independent representative experiment of two are shown. The data shown are the mean ± s.e.m. Where not seen, the s.e.m. bars are smaller than the symbol used.

METHODS

Cloning of GS-MMP-GS linker into *Eco*RI-*Not*I sites of pcDNA3. A double-stranded deoxyoligonucleotide coding for GGGGSPLGLWAGGGS was designed as follows: sense: 5′-AATTCGGGGGAG GCGGATCCCCGCTCGGGCTTTGGGCGGG AGGGGGCTCAGC-3′; antisense: 5′-GGCCGCT GAGCCCCCTCCCGCCCAAAGCCCGAGC GGGGATCCGCCTCCCCCG -3′. Annealed deoxyoligonucleotides were cloned into pcDNA3 cleaved with *Eco*RI and *Not*I. The correct clone was named GS-MMP-GS.

Construction of TGF-β-LAP at the N terminus followed by GS-MMP-GS and mature IFN-β. Human TGF-β LAP as 5′ unit with its signal peptide and with *Hin*dIII and *Eco*RI ends was cloned by PCR from plasmid TGF-β-Babe neo[49]. The following primers were used: sense, 5′-CCAAGCTTATGC CGCCCTCCGGGCTGCGG-3′; antisense, 5′-CCG AATTCGCTTTGCAGATGCTGGGCCCT-3′. The 820-base pair (bp) product was cloned into GS-

MMP-GS plasmid cut with *Hin*dIII and *Eco*RI. The clone was named TGF-β-GS-MMP-GS linker. Mature IFN-β with 5′ *Not*I and 3′ *Xba*I sites was synthesized by PCR from clone Aphrodite[50] using the following primers: sense, 5′-CGCGGCCGCAATCAACTATAAGCAGCTCCAG-3′; antisense, 5′-GGTC TAGATCAGTTTTGGAAGTTTCTGGTAAG-3′. It was then cloned into *Not*I and *Xba*I sites of TGF-β-GS-MMP-GS linker plasmid. The name of the correct clone was LAP-IFN-β.

Construction of IFN-β at the N terminus followed by GS-MMP-GS and mature TGF-β-LAP. Pre-IFN-β with signal peptide and without stop codon was synthesized by PCR as above using the following primers: sense, 5′-CCAA GCTTATGAACAACAGGTGGATCCTC-3′; antisense, 5′-CCGAATTCGTTTT GGAAGTTTCTGGTAAG-3′. The DNA product was cloned into plasmid pcDNA3 GS-MMP-GS digested with *Hin*dIII and *Eco*RI. The clone was named IFN-β + GS-MMP-GS linker. Mature TGF-β-LAP with stop codon was synthesized by PCR as above using the following primers: sense, 5′-CGCGGCCGC ACTATCCACCTGCAAGACTATC-3′; antisense, 5′-GGTCTAGATCAGCTTT GCAGATGCTGGGCCCT-3′. The DNA fragment was cloned into plasmid IFN-β-GS-MMP-GS linker digested with *Not*I and *Xba*I to obtain the fusion protein. The correct clone was named IFN-β-LAP.

Cloning of porcine LAP in front of IFN-β. The doubly mutated porcine TGF-β1 cDNA Cys223 and Cys225 to Ser223 and Ser225 as plasmid pPK14 (ref. 13), was kindly provided by P.J. Wirth, National Institutes of Health, Bethesda, Maryland, USA. Cloning was done by PCR, using the following set of primers: sense (starting at signal peptide), 5′-CGCCCATGGCGCCTTCGGGGCCT-3′ (this primer has a modified sequence around the initiator ATG to create a *Nco*I site); antisense, 5′-CCGAATTCGCTGTGCAGGTGCTGGGCCCT-3′. The PCR product was blunted with the Klenow fragment of DNA polymerase, cut with *Eco*RI, and then cloned into LAP-IFN-β plasmid that had been cut with *Hin*dIII (filled-in) and cut with *Eco*RI (replacing human LAP). The clone was named PorcLAP-IFN-β.

Permanent transfection into DHFR⁻ CHO cells. DHFR⁻ CHO cells were maintained and permanently transfected with plasmids (20 μg) expressing LAP-IFN-β or IFN-β-LAP, each linearized with *Pvu*I and ligated separately with *Pvu*I-cut pSV_2DHFR (1 μg)[51]. The DNA was added as 1 ml calcium phosphate coprecipitate on CHO cells and selected as described[51] with the addition of 1 mg/ml G418. For gene amplification, cells were selected with MTX at 50 nM (LAP-IFN-β) or 12.5 nM (IFN-β-LAP), respectively. Further amplification of LAP-IFN-β in 500 nM MTX facilitated its purification by affinity chromatography.

Transient transfection into monkey COS-7 cells. Plasmid DNA (20 μg) was transfected as described above, in duplicate, into 0.5×10^6 COS-7 cells. The supernatants were collected for IFN assay 48 h after glycerol shock.

IFN-β biological assay. Mouse IFN-β biological activity was assessed on mouse LTK or L929 cells by inhibition of the cytopathic effect of EMC virus (kindly provided by I. Kerr, Imperial Cancer Research Fund, London) as described[50]. Where indicated, serum-free CHO cell supernatants were concentrated using filters with a cut off of 30.0 kDa.

Metabolic labeling of CHO cells. Permanently transfected or nontransfected CHO cells were washed with cysteine- and methionine-free medium containing 10% dialyzed FBS and supplemented with thymidine, glutamine, penicillin, streptomycin and L-proline. Labeling was carried out overnight or for 48 h in the presence of a [^{35}S]methionine-[^{35}S]cysteine mix at 1 Ci/mmol using 250 mCi in 5 ml medium. Supernatants were collected and supplemented where indicated with serine-protease inhibitors (SPI) (pepstatin-A at 10 μg/ml, aprotinin at 1 μg/ml, chymostatin at 10 μg/ml, leupeptin at 10 μg/ml and AEBSF (4-(2-aminoethyl) benzene sulfonyl fluoride, HCl) at 200 μM.

Immunoprecipitation. ^{35}S-labeled supernatants were precleared with protein G–Sepharose in PBS with 0.1% NP-40. Supernatants were incubated with goat anti–human-LAP antibody (at 0.9 μg/ml; R&D Systems) or monoclonal rat anti–IFN-β (monoclonal 7F-D3 at a dilution of 1:250; Yamasa) for 3–4 h at 4 °C. The antigen-antibody complexes were bound to protein G–Sepharose overnight at 4 °C and washed three times with 5 ml 0.1% NP-40 in PBS. Proteins bound to beads were directly resuspended in Laemmli loading buffer (Tris-HCl 0.1 M, pH 6.8, 5% SDS wt/wt, 0.1% bromophenol blue wt/vol, 50% glycerol wt/vol) or used in MMP reactions before electrophoresis on a 10% SDS-polyacrylamide gel. Alternatively, supernatants were treated with MMPs as described below and then immunoprecipitated. Gels were fixed and treated with 1 M sodium salicylate before being dried and exposed to autoradiography.

MMP digestion. Recombinants pro-MMP9 (kindly provided by R. Fridman, Wayne State University, Detroit, Michigan, USA) or active MMP1 and MMP3 (kindly provided by H. Nagase, Kennedy Institute of Rheumatology, Imperial College, London) either were incubated overnight at 37 °C with immunoprecipitated supernatants from CHO cells in 20 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 140 mM NaCl and 0.1% Brij 35 in 50 μl at 1 μg/ml or were directly added to cell supernatants (at 4 μg/ ml). APMA at 10 μM was used in certain experiments to activate pro-MMP9 overnight at 37 °C[52].

Purification of LAP-IFN-β by affinity chromatography. Goat polyclonal, affinity-purified anti–human LAP antibody (500 μg; R&D Systems) was bound to CarboLink (Perbio Science) by chemical crosslinking using manufacturer's instructions. We obtained 70% crosslinking of the antibody in 2 ml of resin.

Supernatant (168 ml) from CHO cells expressing LAP-IFN-β and amplified with 500 nM MTX in CD medium (Invitrogen Life Technologies) was loaded onto the column. Before loading, using fast performance liquid chromatography (AKTA), the CD medium was supplemented with 5 mM EDTA (pH 8.0) at a flow rate of 0.22 ml/min. Bound material was washed with 10 mM phosphate, 140 mM NaCl and 5 mM EDTA, pH 6.8, and eluted with 100 mM glycine and 140 mM NaCl, pH 2.5. Fractions of 1 ml were collected and their pH neutralized with 50 μl of 1 M phosphate buffer, 5 mM EDTA, pH 8.0, and were later dialyzed against PBS at 4 °C. After analysis by 10% SDS-PAGE on Hybond-P membranes (Amersham Biosciences), the presence of LAP-IFN was assessed by western blotting using 1% casein (Fisher Scientific) as blocking agent in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) with 0.2 % Triton X-100 and 0.05% Tween 20 (vol/vol). Next, the blots were probed with a primary rat anti-IFN-β antibody (at 1/1,000) and a secondary goat anti–rat IgG $F(ab')_2$-HRP antibody (at 1:1,000; ImmunoPure, Perbio Science). Using anti-LAP (at 1:1,000; R&D Systems), westerns were subjected to detection using as secondary antibody mouse anti-goat-IgG–horseradish peroxidase conjugate (at 1:1,000; Santa Cruz Biotechnology). The western blots were developed using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) and exposed to autoradiography using Hyperfilm (Amersham Biosciences). Films were developed using an AGFA Curix 60 developer (Gevaert).

Protein iodination and pharmacokinetic studies. Purified LAP-IFN (7 μg) was iodinated in PBS using iodogen and ^{125}NaI (3.7 GBq/ml; Amersham Biosciences) in a final volume of 400 μl for 10 min in ice. The reaction was quenched with 2.5 mM L-tyrosine and after 10 min BSA was added to a final concentration of 0.2 mg/ml. The solution was centrifuged through a 10-ml packed G-25 Sephadex spin column equilibrated in PBS with 1 mg/ml BSA at 5,000*g* for 1 min. The protein was eluted in 1 ml and had a specific activity of 30,312 c.p.m./μl as assessed by counting on a γ counter (LKB Wallac 1282, Pharmacia). The labeled protein was assessed for integrity and correct molecular weight by SDS-PAGE and autoradiography.

Wistar rats (A. Tuck & Son) ($n = 2$) weighing more than 300 g were injected intraperitoneally with either 400 or 500 μl of iodinated protein and blood samples taken by tail vein puncture at the times indicated. All samples were weighed to correct c.p.m. per sample size. At the end of the experiment organs were also removed, weighed and counted as above. Pharmacokinetic data for each rat were analyzed using the program Kinetica (http://www.innaphase.com). The half-life reported is the average of the data obtained from each rat.

CIA and plasmid DNA injection. All animals used in this study were kept according to Institutional and Home Office guidelines. DBA/1 mice were immunized with collagen type II (CII) as described[53] and 3 weeks later were boosted with 100 μg CII in incomplete Freund's adjuvant. We injected mice with 100 μg plasmid DNA in PBS intramuscularly at the onset of arthritis (arthritis score = 1). Subsequently, mice were scored every other day by a blinded observer for clinical arthritis. Each paw was given a clinical score from 0 to 3 as follows: 0 = normal; 1 = slight swelling and/or erythema; 3 = pronounc-

ed edematous swelling; 3 = ankylosis. Inflammation was assessed by hind paw swelling measured with calipers as previously described[52]. Statistical analysis was done by the nonparametric Mann-Whitney test.

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The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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