Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 657–667
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Decoy receptor 3 (DcR3) is proteolytically processed to a metabolic fragment having differential activities against Fas ligand and LIGHT

Victor J. Wroblewski[a,*], Derrick R. Witcher[b], Gerald W. Becker[b], Kelly A. Davis[a], Shenshen Dou[b], Radmila Micanovic[b], Christy M. Newton[b], Timothy W. Noblitt[b], John M. Richardson[b], Ho Yeong Song[b], John E. Hale[b]

[a]*Department of Drug Disposition Development/Commercialization, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA*
[b]*Department of Bioresearch Technologies/Proteins, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA*

Received 19 April 2002; accepted 23 July 2002

Abstract

Fas ligand (FasL) and Fas receptor are members of the tumor necrosis factor (TNF) receptor and ligand family that play an important role in regulating apoptosis in normal physiology. Decoy receptor 3 (DcR3) is a novel member of the TNF receptor superfamily, which binds to and blocks the activities of the ligands FasL and LIGHT. We have demonstrated that DcR3 was degraded rapidly to a major circulating metabolic fragment after subcutaneous administration in primates and mice. This fragment was also generated in subcutaneous tissue homogenate *in vitro*. Mass spectrometry and N-terminal sequencing indicated that DcR3 was proteolytically cleaved between R218 and A219 in the primary sequence to yield the fragment DcR3(1-218). While retaining its ability to bind LIGHT and inhibit LIGHT-mediated activities, DcR3(1-218) no longer bound FasL and did not inhibit FasL-mediated apoptosis *in vitro*. The primary sequence of DcR3 was molecularly engineered, changing the arginine residue at position 218 to glutamine to generate an analog, DcR3(R218Q), which we termed FLINT (LY498919). We demonstrated that FLINT was more stable to proteolytic degradation *in vitro* and *in vivo* and maintained its activity against both soluble FasL and soluble LIGHT *in vitro*. As a result, the modification in the sequence of DcR3 to produce FLINT (LY498919) should result in a pharmacologically superior molecule in the therapeutic intervention of diseases in which the pathogenesis is linked to FasL-mediated apoptotic or inflammatory events.

Keywords: Fas ligand; LIGHT; Decoy receptor 3; Apoptosis; TNF

1. Introduction

The members of the TNF receptor superfamily have been shown to be involved in diverse biological activities such as immune regulation, cell survival, cell death, and the regulation of cell proliferation and differentiation [1–4]. FasL and Fas receptor are members of the TNF receptor and ligand family that play an important role in regulating apoptosis in normal physiology. Prior to recent published reports, we also identified a novel member of the TNF receptor superfamily, called DcR3 (TR6), from a search of an expressed sequence tag database [5]. The processed, soluble form of DcR3 is a 271 amino acid polypeptide having one N-linked glycosylation site. In agreement with previous literature reports [5,6], we have demonstrated that DcR3 binds both FasL and LIGHT and blocks both soluble FasL (sFasL)-mediated apoptosis of human Jurkat cells and the LIGHT-mediated inhibition of HT-29 cell growth, in a dose-dependent fashion. These data are consistent with a potential role for DcR3 as an inhibitor of signaling via these receptor pathways *in vivo*.

While Fas/FasL-mediated apoptosis is believed to be important in the maintenance of normal physiological processes, excessive or inappropriate apoptosis induced by the Fas/FasL system has been proposed to be a key factor in the pathogenesis of autoimmune disease, cancer,

* Corresponding author. Tel.: +1-317-276-9306; fax: +1-317-276-4218.
E-mail address: wroblewski_victor@lilly.com (V.J. Wroblewski).

Abbreviations: TNF, tumor necrosis factor; FasL, Fas ligand; FLINT, Fas ligand inhibitor protein; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; RP-HPLC, reversed-phase high performance liquid chromatography; PVDF, polyvinylidenedifluoride; HVEM, herpesvirus entry mediator; DcR3, decoy receptor 3; PCR, polymerase chain reaction; ACN, acetonitrile; TFA, trifluoroacetic acid; IFN, interferon.

0006-2952/02/$ – see front matter
doi:10.1016/S0006-2952(02)01612-X

and diseases of the liver, lung, kidney, and central nervous system [7–9]. In addition, the interaction of LIGHT with HVEM/TR2 or LTβR may also be involved in regulating apoptosis and modulating functions of the immune system [5,10,11]. More recently, TL1A (endothelial cell-derived TNF-like factor), which acts as a T-cell costimulator, has been shown to be a ligand for DcR3 [12]. The presence of soluble forms of FasL, Fas, other TNF ligands, and antagonistic decoy receptors suggests that these processes, which are integral to normal physiology, are under tight biological regulation. As such, inhibition of apoptotic cell death via disruption of the FasL-, LIGHT-, or TL1A-mediated signaling pathways may be envisioned as a novel pharmacological approach in the therapy of a number of human diseases.

The pharmacokinetics and distribution of DcR3 were studied in small animal models and primates as part of its preclinical development program. After subcutaneous administration, DcR3 was degraded rapidly to a major circulating metabolic fragment. This fragment was also generated using *in vitro* systems, and characterized using mass spectrometry and N-terminal sequencing. DcR3 was shown to be proteolytically cleaved between R218 and A219 in the primary sequence, yielding the circulating fragment DcR3(1-218). While retaining its ability to bind LIGHT and inhibit LIGHT-mediated activities, DcR3(1-218) no longer bound FasL and did not inhibit FasL-mediated apoptosis *in vitro*. The primary sequence of DcR3 was molecularly engineered, changing the arginine residue at position 218 to glutamine to generate an analog, DcR3(R218Q), which we termed FLINT (LY498919). This variant is more resistant to proteolytic degradation *in vitro* and *in vivo* and maintains its activity against both sFasL and sLIGHT *in vitro*.

2. Materials and methods

2.1. Engineering vector for the expression of DcR3 and analogs in mammalian cells

A bicistronic expression vector was constructed by insertion of an "internal ribosome entry site"/enhanced green fluorescent polypeptide PCR fragment into the mammalian expression vector pGTD [13]. This new vector, designated pIG1, contained the following sequence landmarks: an E1a-responsive GBMT promoter [14,15]; a unique *Bcl*I cDNA cloning site; the IRES sequence from encephalomyocarditis virus (EMCV); the eGFP (Clontech) coding sequence [16]; the SV40 small "t" antigen splice site/polyadenylation sequences; the SV40 early promoter and origin of replication; the murine dihydrofolate reductase coding sequence; and the pBR322 ampicillin resistance marker/origin of replication.

Based on the human DcR3 cDNA sequence, the forward and reverse PCR primers were synthesized bearing the *Bcl*I restriction site at their respective 5′ and 3′ ends. These primers were used to amplify the DcR3 analog cDNA by PCR. The human DcR3 cDNA orientation and nucleotide sequence were confirmed by restriction digestion and double-stranded sequencing of the insert.

2.2. Isolation of a high-producing DcR3 analog clone from AV12 RGT18 transfectants

The recombinant plasmid carrying the *DcR3* gene encoded resistance to methotrexate. In addition, the construct contained a gene encoding a fluorescent polypeptide, GFP, on the same transcript and immediately 3′ to the *DcR3* gene. Transfected cells resistant to methotrexate (250 nM) were subjected to FACS cell sorting, and cells having fluorescence values in the top 5% of the population were collected. The high fluorescence pools were subjected to three successive sorting cycles. Pools or clones expressing DcR3 at the highest level judged from Coomassie-stained SDS–PAGE gels were used for scale-up and DcR3 purification.

2.3. Production and purification of DcR3 and DcR3(R218Q)

Production of DcR3 was carried out by first growing the stable clone pIG1-DcR3-containing AV12 RGT 18 cells in several 10-L spinners. Medium containing DcR3 was adjusted to 0.1% CHAPS and concentrated in an Amicon ProFlux M12 tangential filtration system to 350 mL. The concentrated medium was adjusted to pH 6.0 and passed over an SP Sepharose Fast Flow (Pharmacia, 50 mL), followed by fractionation of the DcR3-containing pool by RP-HPLC on a Vydac C4 column (1 × 15 cm). Fractions containing DcR3 were pooled, concentrated under vacuum, and applied to a Superdex 75 (Hi Load 16/60, Pharmacia) size exclusion column. Fractions containing DcR3 were analyzed by SDS–PAGE and found to be greater than 95% pure. The N-terminal sequence of DcR3 was confirmed on the purified polypeptide.

2.4. Production and purification of the DcR3(1-218)

DcR3 purified from AV12 RGT 18 cells was incubated with thrombin at an enzyme to substrate ratio of 1:100 (w/w) for 3 hr at room temperature. The reaction solution was then dialyzed against 20 mM MOPS, 0.1% CHAPS, pH 6.5, and fractionated on an SP Sepharose column at a flow rate of 1 mL/min. The bound metabolite (amino acids 1-218) was eluted, and fractions were analyzed by SDS–PAGE and mass spectrometry. Fractions containing only the DcR3(1-218) were pooled and concentrated in a Millipore Ultrafree centrifugal filter. The concentrated DcR3(1-218) was again analyzed by SDS–PAGE and mass spectrometry to assess purity. The N-terminal of the DcR3 metabolite was confirmed by Edman sequencing.

2.5. Soluble LIGHT (sLIGHT-Flag)

The sequence encoding the putative extracellular domain of LIGHT (amino acids 74-240) was subcloned into the pFlag-CMV-1 expression vector. The resulting plasmid was transiently transfected into 293T cells using Lipofectamine Plus (Gibco-BRL). sLIGHT-Flag was purified from the transfected 293T supernatant using an anti-Flag mAb affinity column (Eastman Kodak). sLIGHT-Flag (21 kDa) was detected by western blot analysis using the anti-Flag Bio M2 antibody (Sigma F-9291). sLIGHT used in the HT-29 proliferation assay with FLINT was purchased from R&D Systems.

2.6. Radioiodination

DcR3 or FLINT was radiolabeled using the Iodobeads® iodination reagent (Pierce). The radiolabeled product was separated from unincorporated iodide using a PD-10 column. The final specific activity of the tracer ranged from approximately 5 to 15 mCi/mg. ^{125}I-DcR3 and -FLINT were >90% precipitable in 15% trichloroacetic acid. On RP-HPLC, ^{125}I-DcR3 and -FLINT eluted as a single major protein peak having a retention time of 35 min.

2.7. Animal studies

^{125}I-DcR3 or ^{125}I-FLINT was administered to male CD-1 mice (20–25 *g*) as single subcutaneous doses ranging from 0.18 to 8 mg/kg (5–15 μCi/mouse). DcR3 or FLINT was also administered to male cynomolgus monkeys (*Macaca fascicularis*) as a single subcutaneous dose of 1 mg/kg. Blood samples were collected into EDTA tubes containing protease inhibitor fluid (Boehringer Ingleheim) for preparation of plasma. Plasma samples were fractionated on RP-HPLC as described below.

2.8. RP-HPLC

Plasma and *in vitro* tissue samples were fractionated on a Vydac C_4 (4.6 × 150 mm) column. Proteins were eluted off of the column with a linear gradient of 15% A/85% B to 55% A/45% B in 40 min at a flow rate of 1 mL/min (Buffer A = ACN/0.08% TFA, Buffer B = H_2O/0.1% TFA). Fractions (0.5 or 1 mL) were collected, and radioactivity was determined directly in a gamma counter. Unlabeled protein fractions were concentrated to dryness in a Speed Vac (Savant Instruments) prior to resuspension in 0.5 mL PBS/0.1% Tween-20/1% BSA for analysis by ELISA (below).

2.9. ELISA

Antiserum was produced in male New Zealand white rabbits immunized with DcR3 in complete Fruend's adjuvant. Polyclonal antibodies were prepared by affinity chromatography on Protein A. The polyclonal antibodies were immunopurified on an affinity column prepared using DcR3. Immunopurified antibodies were prepared from individual animals and used directly as capture antibodies or biotinylated for use as the detection antibody in a sandwich assay format. Wells of a 96-well microtiter plate were coated overnight at 4° with purified polyclonal antibody TKD-028 (5 μg/mL, 0.1 mL/well). After washing, standards and samples were added in a volume of 100 μL/well and incubated at room temperature for 2 hr. After washing, biotinylated antibody TKD-076A was added (1:4000 dilution, 100 μL/well) and incubated at room temperature for 1 hr. After washing, streptavidin-alkaline phosphatase (Boehringer Ingleheim) was added (1:1000 dilution, 100 μL/well) and incubated at room temperature for 1 hr. Detection was accomplished with the addition of Attophos™ substrate (50 μL/well). Fluorescent intensity was measured at 15-min intervals at room temperature in a Biolumin microplate reader (Molecular Dynamics). The assay had a standard curve range from 0.125 to 20 ng/mL. The limit of quantitation was determined to be 0.5 ng/mL.

2.10. Immunoprecipitation/western blotting

Control mouse plasma, DcR3 added to mouse plasma (100 ng/mL), and plasma samples from DcR3-treated mice (0.5 mL each) were incubated for 16 hr (4°) with 0.05 mL of a 1:50 dilution of rabbit anti-DcR3 antisera (No. 1494). Protein-A agarose (0.05 mL of a 50% slurry) was added, and the incubation was allowed to proceed for an additional 1 hr at room temperature. Samples were sedimented at 16,000 *g* for 5 min in a microfuge (Eppendorf 5415C; Savant Instruments). The supernatant was decanted, and the pellet was washed three times with 1 mL of PBS. The protein-A agarose pellet was then resuspended in SDS–PAGE buffer with 2-mercaptoethanol and boiled for 10 min. After sedimenting the agarose pellet in a microfuge, the supernatant was applied to the wells of a 16% polyacrylamide gel. After electrophoresis, proteins were transferred electrophoretically to a PVDF membrane. After blocking, the membrane was incubated for 1.5 hr with a 1:500 dilution of rabbit anti-DcR3 antisera (No. 1494). Immunoreactive bands were detected with goat anti-rabbit horseradish peroxidase conjugate.

2.11. In vitro studies

Sections (1 cm × 1 cm) of skin and subcutaneous tissue were dissected from CD-1 mice under anesthesia. The subcutaneous layer and fat were removed with a straight edge razor and homogenized with a polytron homogenizer (Brinkmann) in 10 mM PBS (pH 7.2) to give a 30% (w/v) homogenate. The homogenate then was sedimented at 1000 *g* for 5 min at 4° to remove debris. DcR3 (2.6 μM)

was incubated with a 3% homogenate for 24 hr at 37°. The metabolic profile was resolved by RP-HPLC or using a 16% acrylamide gel with detection by immunoblot analysis as previously described. The metabolite was isolated and purified from the *in vitro* reactions using immunoprecipitation followed by RP-HPLC fractionation. Purified metabolite(s) was identified using MALDI-TOF and electrospray mass spectrometry and N-terminal Edman sequencing (see below).

2.12. Whole blood incubations

^{125}I-DcR3 or ^{125}I-FLINT (10 ng) was added to serum tubes. CD-1 mouse blood (1 mL) was collected directly into these tubes via cardiac puncture. Samples were allowed to clot for 1 hr at 37°. Serum was prepared by centrifugation (3000 *g*, 15 min, 4°) and directly analyzed by RP-HPLC.

2.13. Incubation with bovine thrombin

^{125}I-DcR3 or ^{125}I-FLINT (10 ng/mL) was incubated with bovine thrombin (5.7 μg/mL) in 20 mM Tris, 150 mM NaCl, pH 7.4, for 1 hr at 37°. Samples were directly analyzed by RP-HPLC. Additionally, unlabeled DcR3 and FLINT (1 μg) were incubated with bovine thrombin (10 ng) in 20 mM Tris, 150 mM NaCl, pH 7.4, or PBS, 0.5 M NaCl, 10% glycerol, and thrombin at 37° for up to 4 hr. Reaction mixtures were analyzed by SDS–PAGE.

2.14. MALDI-TOF mass spectrometry

MALDI-TOF spectra were obtained on a Micromass TOFSpecE using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix at a concentration of 10 mg/mL in 0.1% (v/v) TFA/50% (v/v) ACN. The mass spectrometer was calibrated with trypsinogen and carbonic anhydrase (Sigma). HPLC fractions containing DcR3 and DcR3 fragments were spotted onto a MALDI target, matrix added and spectra acquired at laser course and fine settings of 80 and 50, respectively.

2.15. HT-29 proliferation assay

HT-29 cells (5×10^3 cells/well) were plated into 96-well tissue culture treated plates (Costar 3596) in 50 μL assay medium [McCoy's 5 alpha medium/10% fetal bovine serum and 50 units/mL of rhIFN-γ (R&D Systems)]. Serial dilutions of purified sLIGHT-Flag with or without 192 nM DcR3(1-218) in assay medium were added to give a final well volume of 100 μL. Alternatively, cells were plated in assay medium containing 50 ng/mL of sLIGHT (from R&D Systems) in the presence of increasing concentrations of DcR3 or FLINT. Cells were incubated for 96 hr at 37°/5% CO_2 and pulsed with 1 μCi [^{3}H]thymidine/well for 3 hr. Cells were then harvested, and radioactivity was determined by β-scintillation counting.

2.16. Jurkat cell apoptosis assay

Jurkat cells (ATCC) were seeded in a 96-well plate at 5×10^4 cells/well in a volume of 25 μL. Human recombinant FasL (Alexis Biochem) was added in 25 μL to a final concentration of 100 ng/mL. DcR3, FLINT, or DcR3(1-218) was added to each well to a final concentration of 1, 0.5, 0.25, 0.125, 0.06, and 0.03 μg/mL in a volume of 50 μL. The total volume of each well was 100 μL. All dilutions were made in RPMI medium containing 10% fetal bovine serum (GIBCO BRL). Cells were incubated overnight at 37°. Apoptosis was measured using the Cell Titer 96-well proliferation assay kit (Promega).

3. Results

3.1. Proteolytic metabolism of DcR3

After subcutaneous administration of DcR3 to mice, SDS–PAGE/western blotting of plasma demonstrated the presence of a circulating fragment having an apparent molecular weight of 27–30 kDa (Fig. 1A). Additionally, RP-HPLC analysis of plasma samples from mice that were administered a subcutaneous dose of ^{125}I-DcR3 indicated that the majority of protein-associated radioactivity at all time points examined corresponded to a metabolic fragment (peak at fraction 32) of DcR3 (Fig. 2A). A fragment having the same retention characteristics on

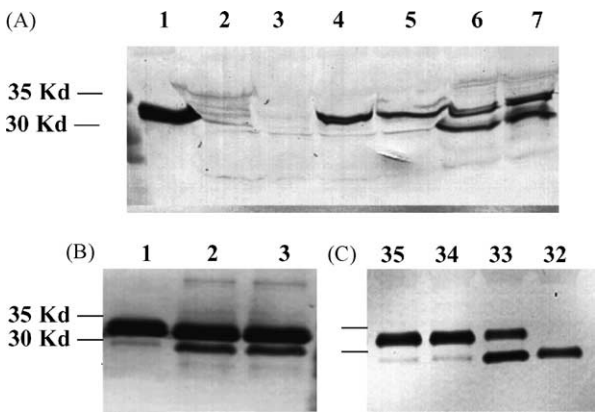

Fig. 1. Proteolytic degradation of DcR3 *in vivo* and in tissue homogenates. Panel A: DcR3 was administered to CD-1 mice by subcutaneous injection. Plasma obtained 4 hr after dosing was immunoprecipitated using anti-DcR3 polyclonal antisera; immunoprecipitates were fractionated on SDS–PAGE, and immunoblots were developed with anti-DcR3 antibodies. Lane 1, DcR3 (100 ng); lanes 2 and 3, control mouse plasma; lanes 4 and 5, DcR3 (100 ng) added to control mouse plasma; lanes 6 and 7, plasma from DcR3-treated mice. Panel B: SDS–PAGE/immunoblot from a 24-hr incubation of DcR3 with subcutaneous tissue homogenate from CD-1 mice. Lane 1, DcR3 in the homogenate (time zero); lanes 2 and 3, tissue homogenate reactions at 24 hr. Panel C: samples from a 24-hr incubation of DcR3 with subcutaneous tissue homogenates were fractionated by RP-HPLC, and collected fractions (32–35) were analyzed by SDS–PAGE/immunoblotting. Data shown are representative of results obtained in several experiments.

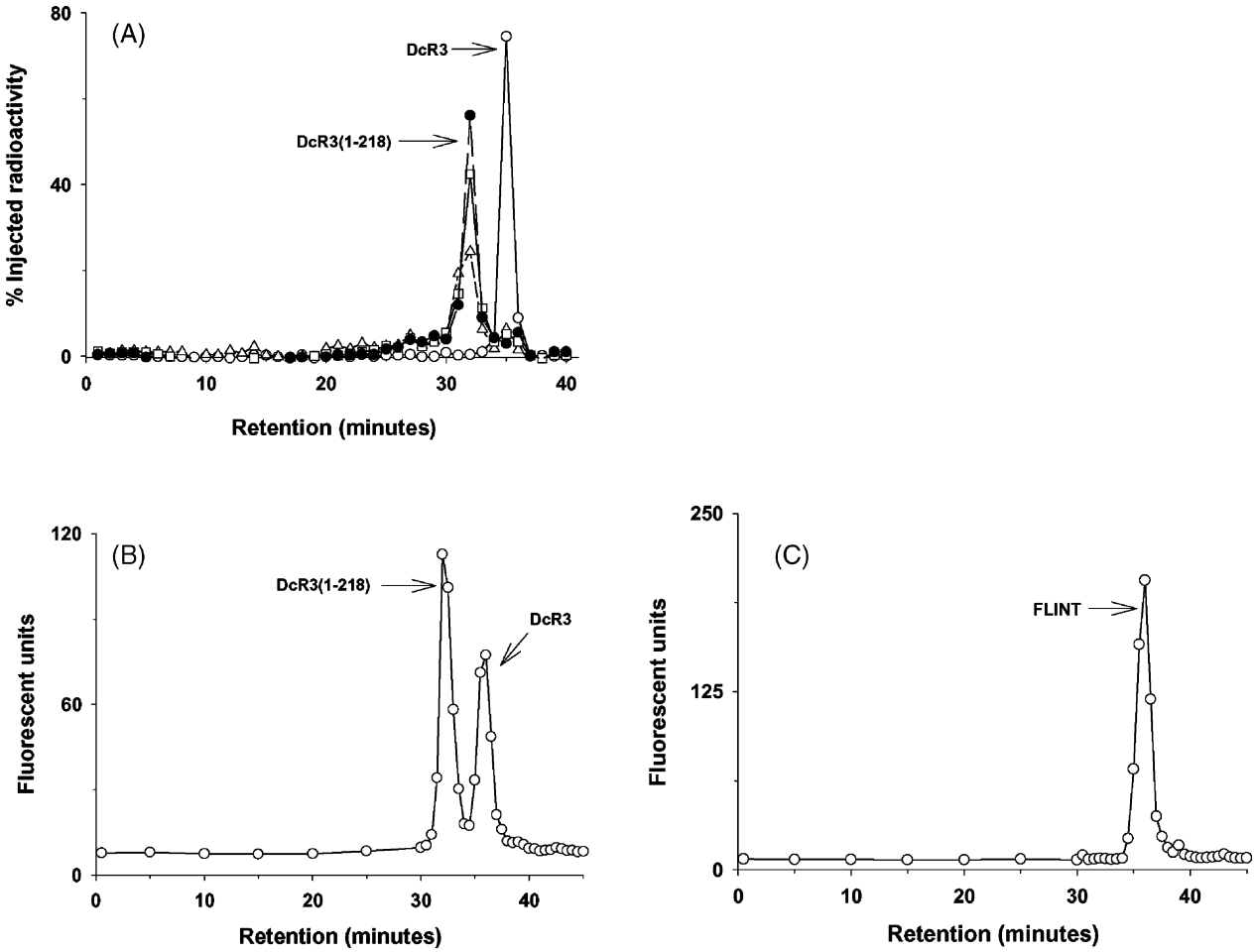

Fig. 2. Resistance of FLINT to proteolytic degradation observed after subcutaneous administration of DcR3. RP-HPLC profile of DcR3-related radioactivity and immunoreactivity following subcutaneous administration of DcR3 to CD-1 mice (panel A) and cynomolgus monkeys (panel B) or FLINT to cynomolgus monkeys. Panel A: radioactive profile of ^{125}I-DcR3 added to plasma (open circles), and in plasma samples obtained 1 hr (open triangles), 4 hr (open squares), and 8 hr (closed circles) after dosing with ^{125}I-DcR3. Panel B: immunoreactivity in plasma 4 hr after subcutaneous administration of DcR3 to monkeys. Panel C: immunoreactivity in plasma 4 hr after subcutaneous administration of FLINT to monkeys. Data shown are representative of results obtained in several experiments.

RP-HPLC was also observed in the circulation after subcutaneous administration of DcR3 to cynomolgus monkeys (Fig. 2B). *In vitro* studies using homogenates of subcutaneous tissue (Fig. 1B) demonstrated that DcR3 was metabolized to a single immunoreactive fragment having an apparent molecular weight similar to that observed after subcutaneous administration. This observation indicated that the metabolic processes occurring in the *in vitro* system were relevant to those occurring at the subcutaneous site after administration of DcR3 to animals. SDS–PAGE/western blotting of RP-HPLC fractions from the *in vitro* incubations (Fig. 1C) provided further confirmation that the fragment formed *in vitro* was the same formed *in vivo* after subcutaneous administration. ^{125}I-DcR3 was also degraded to this metabolite when added to whole blood, which was allowed to clot at 37° for 1 hr (Fig. 3A). The degradation in whole blood was also mimicked by the *in vitro* digestion of DcR3 with purified bovine thrombin (Fig. 3A and C). However, we have not been able to demonstrate the cleavage of DcR3 *in vivo* after intravenous administration (data not shown).

3.2. Identification of the DcR3 metabolite

The subcutaneous tissue homogenate system was used to generate enough pure DcR3 metabolite to allow identification using mass spectrometry (Figs. 4 and 5) and N-terminal Edman sequencing (not shown). After *in vitro* incubation, DcR3 and degradation products were immunoprecipitated and fractionated by RP-HPLC. MALDI-TOF mass spectrometry of fraction 32 (Fig. 1C) from the HPLC separation revealed three peaks having masses of 5947, 25972, and 31924. The broad nature of the 26 and 36 kDa peaks is consistent with the presence of N-linked glycosylation on DcR3. The mass at 31924 represents the mass of residual DcR3. The mass at 25972 differed by 5952 from the intact mass, which is similar to the mass of the third observed peak, 5947. The results were consistent with a cleavage of DcR3 between Arg^{218} and Ala^{219}, in the

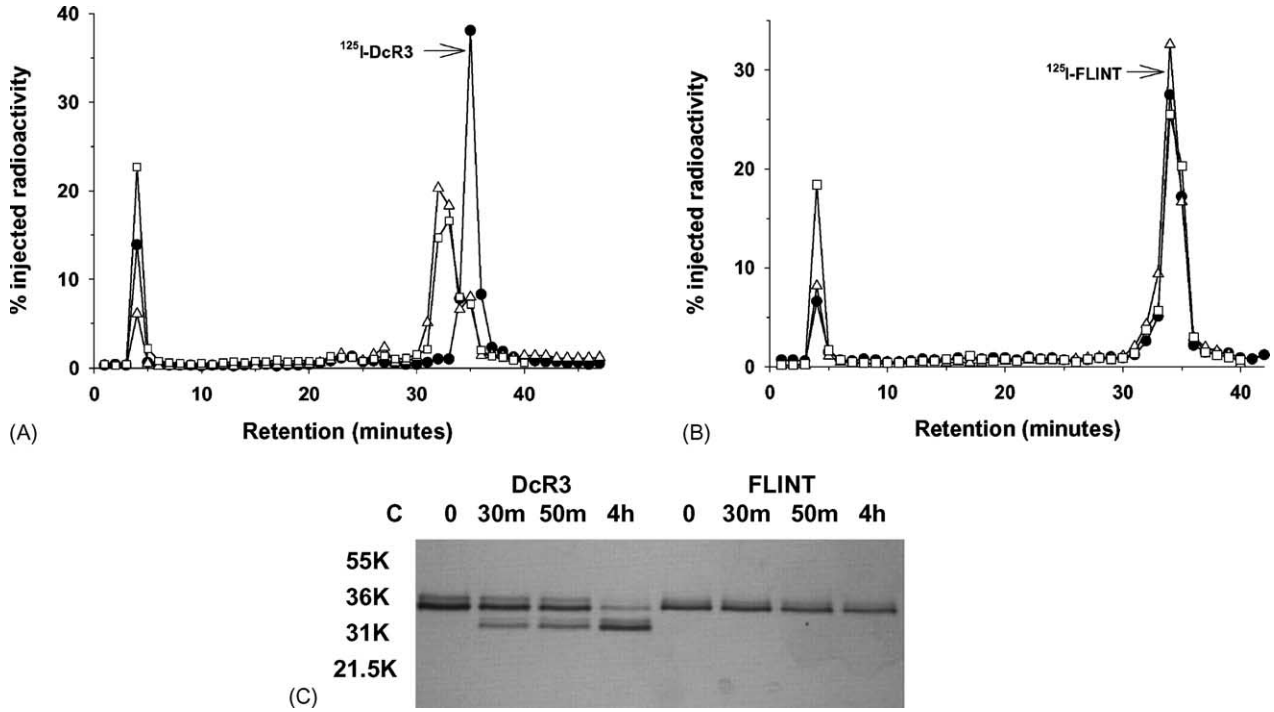

Fig. 3. Resistance of FLINT to proteolytic degradation by serum *ex vivo* and purified bovine thrombin. ^{125}I-DcR3 (panel A) or ^{125}I-FLINT (panel B) was incubated with either whole blood (CD-1 mouse) that was allowed to clot at 37° for 1 hr (open triangles) or purified bovine thrombin for 1 hr (open squares). Samples were then fractionated on RP-HPLC. The profile of control ^{125}I-DcR3 or ^{125}I-FLINT is represented by the closed circles. Panel C: unlabeled DcR3 or FLINT was incubated with bovine thrombin, and samples were analyzed by SDS–PAGE. Data shown are representative of results obtained in several experiments.

primary sequence. Since the MALDI had been calibrated for the higher end of the molecular weight(s) observed, the sample was also analyzed by electrospray mass spectrometry (Fig. 5) to obtain a more accurate mass of the smaller fragment observed using MALDI-TOF mass spectrometry. The spectra indicated that the mass of the peptide was 5984.9, which is virtually identical to the theoretical mass (5986.1) of the 52 amino acid C-terminal fragment (Ala^{219}-His^{271}) of DcR3. Along with Edman sequencing of the fragments, these data confirmed the cleavage of DcR3 between R218 and A219 to yield DcR3(1-218) and the 52 amino acid C-terminal peptide. Based on the determined mass, DcR3(1-218) appears to be a major circulating metabolic fragment formed upon subcutaneous administration of DcR3 to animals. The biological fate of the carboxy terminal 52 amino acid peptide could not be determined *in vivo* as this region of the molecule was not labeled by iodination, due to the absence of a tyrosine, and was not detected in the immunoassay used for measurement of DcR3. Given the molecular weight of this peptide, it is likely that it would be rapidly cleared by renal filtration and subsequently proteolyzed.

3.3. In vitro inhibition of FasL and LIGHT

DcR3 blocked sFasL-mediated apoptosis of human Jurkat cells in a concentration-dependent fashion, with an EC_{50} of approximately 250 ng/mL. In contrast, the metabolic fragment DcR3(1-218) was significantly less active than DcR3 in inhibiting sFasL-mediated apoptosis in this system, demonstrating no inhibition at the EC_{50} determined with DcR3 (Fig. 6A). In contrast, DcR3(1-218) did block the sLIGHT-mediated inhibition of HT-29 cell proliferation (Fig. 7A). These data suggest that while maintaining its ability to bind and potentially inhibit the activities of LIGHT, DcR3(1-218) no longer had the capacity to modulate apoptotic or inflammatory events mediated through the Fas-FasL pathway.

3.4. Characteristics of FLINT

A series of analogs were designed, which eliminated this proteolytic cleavage at Arg^{218}. In one of these analogs the arginine residue was replaced with a glutamine at position 218, yielding DcR3(R218Q). We have named this molecule FLINT. Compared to DcR3, FLINT bound FasL (not shown) and had equivalent potency in blocking sFasL-mediated apoptosis in human Jurkat cells (Fig. 6B). FLINT also bound sLIGHT (not shown) and blocked sLIGHT-mediated inhibition of HT-29 cell growth in a manner consistent with that of DcR3 (Fig. 7B). ^{125}I-FLINT demonstrated complete stability to proteolytic degradation *ex vivo* in whole blood experiments and was resistant to degradation by purified bovine thrombin (Fig. 3B and C). After subcutaneous administration of FLINT in monkeys (Fig. 2C) and mice (not shown), the only form of circulating

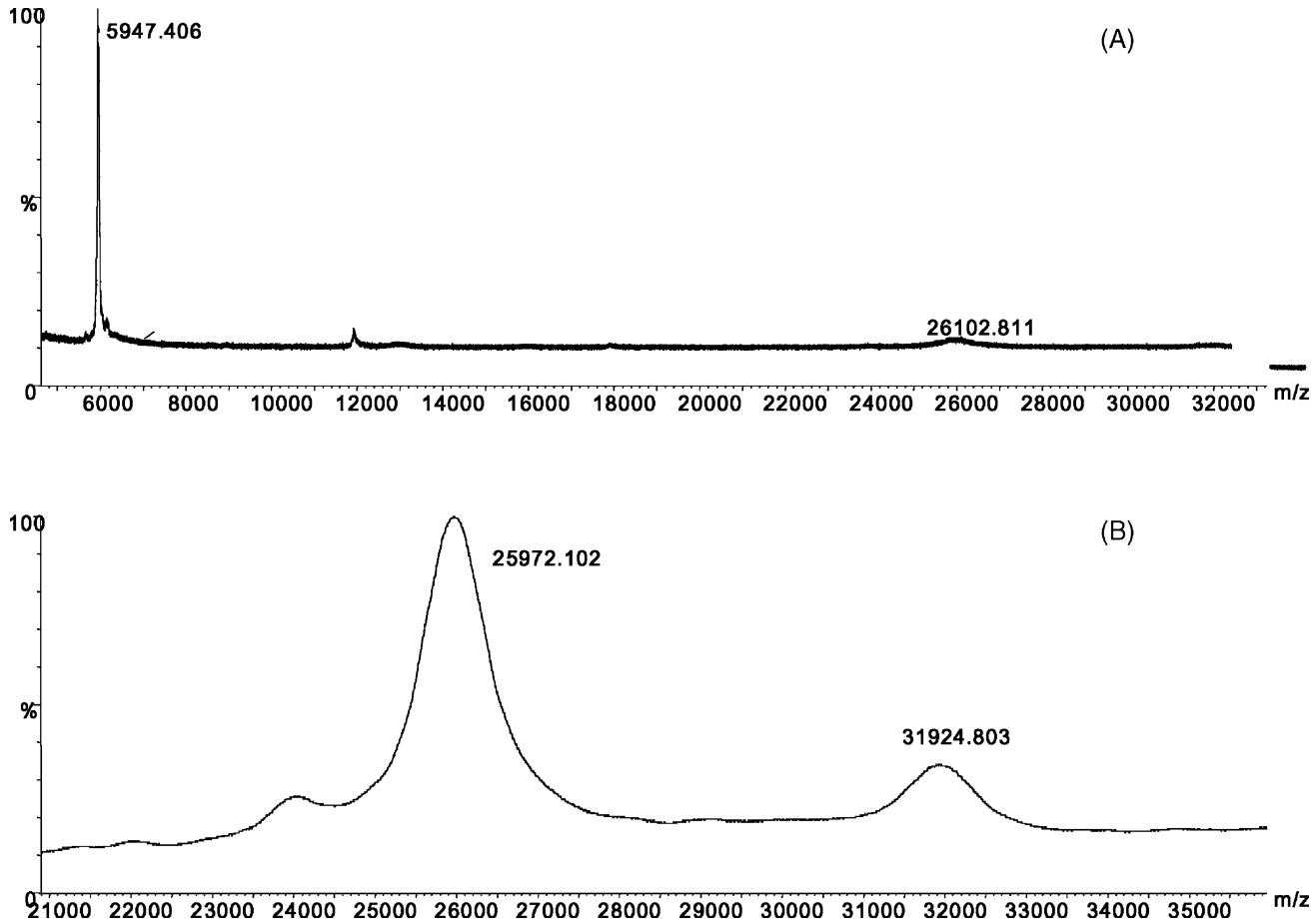

Fig. 4. MALDI-TOF mass spectrometry of DcR3 digested with subcutaneous tissue homogenate. DcR3 was incubated with skin homogenate, subjected to RP-HPLC, and fractions containing intact and degraded DcR3 (determined by western blot analysis; Fig. 1B and C) were analyzed by MALDI-TOF mass spectrometry. Panels A and B represent the spectra of fraction 32 (displayed on different scales). The prominent peak at 5947 in panel A is near the theoretical mass of 5986 for the C-terminal 52 amino acids of DcR3. Panel B represents the mass of residual intact DcR3 (31924) and the large N-terminal fragment (25972), which differs by 5952. The broad nature of these peaks is due to the glycosylation of these proteins.

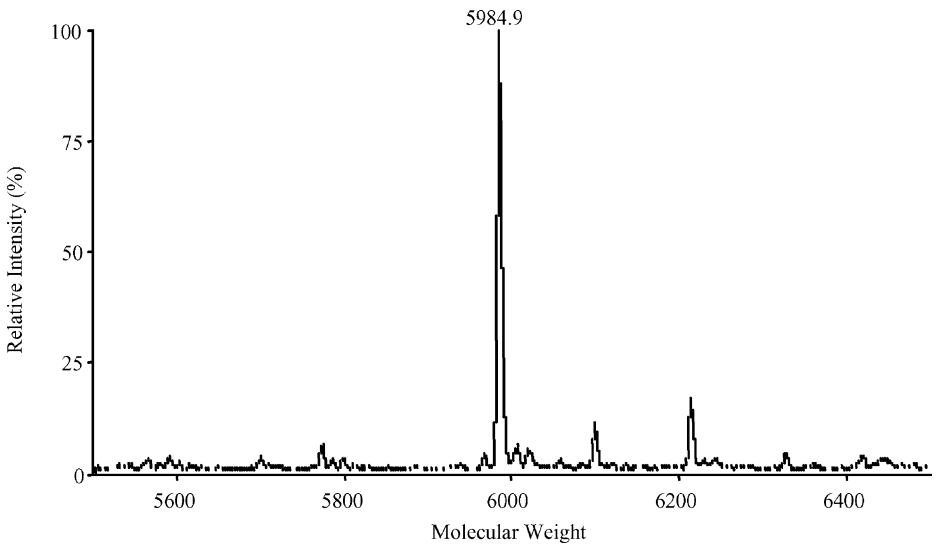

Fig. 5. Electrospray mass spectrometry of the metabolite fraction from the DcR3 skin homogenate incubation. The MALDI-TOF mass spectrometry data suggested that the degradation of DcR3 was occurring at residue 219. To confirm this conclusion, fraction 32 (see Fig. 1C) was subjected to electrospray mass spectrometry under conditions designed to obtain an accurate mass of the N-terminal peptide. The reconstructed spectra are shown, and the mass of 5984.9 is virtually identical to the theoretical mass of 5986.1.

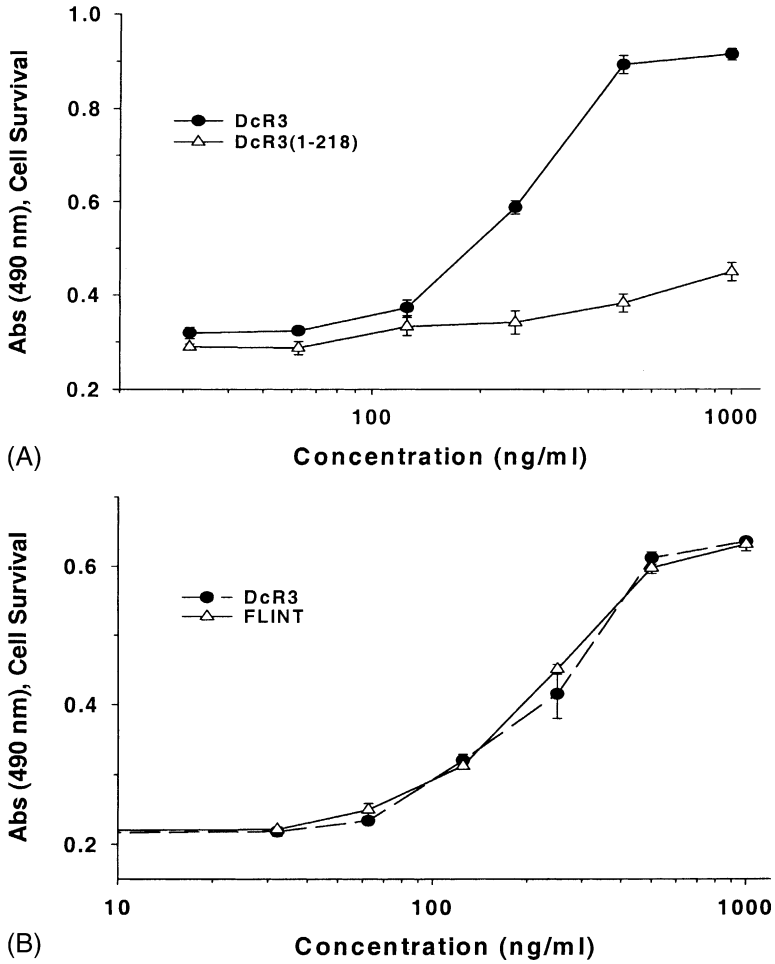

Fig. 6. Effect of DcR3(1-218) on sFasL-mediated apoptosis in human Jurkat cells. Jurkat cells were incubated with sFasL (100 ng) for 24 hr in the presence of increasing concentrations of DcR3, FLINT, or DcR3(1-218). Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay. Panel A: inhibition of FasL-mediated apoptosis in the presence of DcR3 or DcR3(1-218). Panel B: inhibition of FasL-mediated apoptosis in the presence of FLINT or DcR3. Data are means ± SD, N = 3.

immunoreactivity corresponded to intact FLINT, indicating that the molecule was also resistant to the proteolysis observed *in vivo* after administration of DcR3.

4. Discussion

DcR3 binds specifically to FasL, LIGHT, and TL1A and inhibits the activities of these TNF receptor family ligands. These ligand systems have been implicated in modulating cell survival, cell proliferation, and cell death [1–5]. As such, regulation of the expression of mediator molecules such as the soluble forms of FasL, Fas, and antagonistic decoy receptors such as DcR3 may be involved in the tight control of these biological functions which are integral to both normal physiology and pathophysiology [17]. The relative levels of these mediators may act to balance the intensity of processes involved in normal physiological or pathophysiological conditions. Similarly, pharmacologic manipulation of the biological events triggered by either FasL, LIGHT, or TL1A may have therapeutic benefit in human diseases in which the pathogenesis is believed to be related to excessive or inappropriate apoptosis or inflammation [8].

In early preclinical studies, DcR3 was shown to be proteolytically degraded *in vitro* and *in vivo* to a single major metabolic fragment. Using MALDI-TOF and electrospray mass spectrometry techniques, the metabolic fragment was identified from *in vitro* incubations as the N-terminal 218 amino acids of DcR3. The data indicated that position R218-A219 was uniquely sensitive to proteolysis and may be one of the primary sites of degradation of DcR3 when administered *in vivo*. While proteolytic degradation of proteins is often non-limited, leading to complete degradation and inactivation of the target protein via hydrolysis of a multitude of peptide bonds, there are several examples of proteins whose activities are modulated by limited and specific proteolysis. Limited proteolysis may serve to alter the biologic activity of a protein at its cellular site of action, selectively inactivate the protein, or yield the mature activity of a protein. Several growth factors and hormones such as prolactin, growth hormone, parathyroid hormone, and platelet-derived growth factor-BB undergo

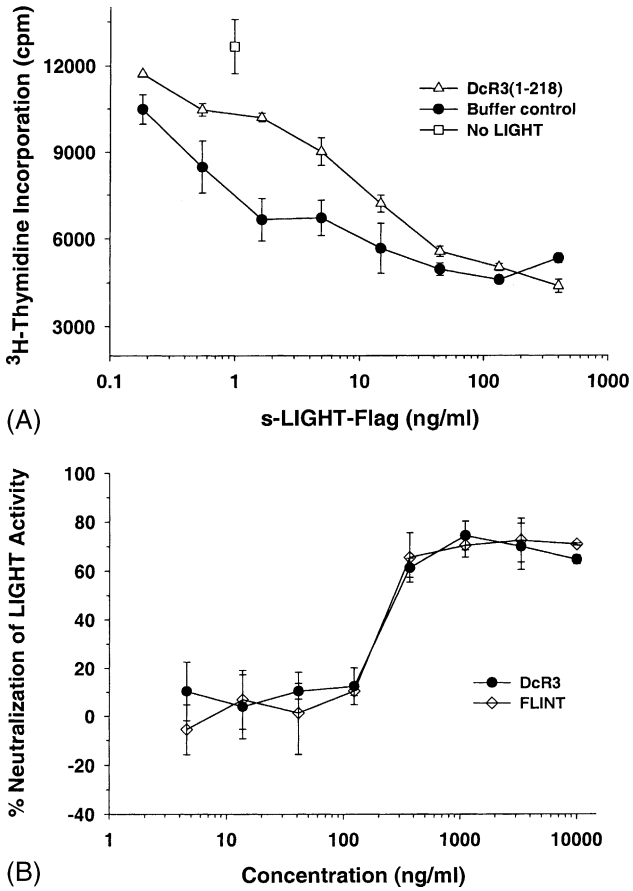

Fig. 7. Effect of DcR3(1-218) on sLIGHT inhibition of HT-29 cell proliferation. Panel A: HT-29 cells were incubated with 50 units/mL of rhIFN-γ and 192 nM DcR3(1-218) for 96 hr in the presence of increasing concentrations of sLIGHT-Flag. Panel B: HT-29 cells were incubated with 50 units/mL of rhIFN-γ and 50 ng/mL of sLIGHT for 96 hr in the presence of increasing concentrations of DcR3 (closed circles) or FLINT (open diamonds). In both panels, proliferation was determined by [^{3}H]thymidine incorporation. Data are means ± SD, N = 3.

limited proteolysis *in vivo* to yield variant molecules that have been shown to have altered, enhanced, or selective biological activities [18–21]. Similarly, minimal truncation of a number of chemokines has been shown to yield molecules having altered receptor specificity and modified inflammatory and antiviral responses [22]. This type of regulation may be necessary to specifically direct the activity of a molecule under various physiological conditions. Given this, we examined whether the limited proteolysis of DcR3 could also have relevance to its biological activities. *In vitro*, we demonstrated that proteolysis of DcR3 at position R218 resulted in a differentiation of the binding activities demonstrated using the intact molecule. DcR3(1-218) did not bind FasL or inhibit FasL-mediated apoptosis, but maintained its ability to bind LIGHT and interrupt activities mediated through this ligand binding pathway. At this time, the interaction of DcR3(1-218) with TL1A is not known. As a member of the TNF receptor family, DcR3 contains four homologous structural and functional domains stabilized by canonical disulfides, designated D1–D4. It is thought that ligands interact with the TNF receptors through D2 and D3. The functional data obtained for DcR3(1-218) would suggest that LIGHT may interact with these two domains; however, it appears that the ability of FasL to bind to DcR3 is modulated by more that just D2 and D3. The data indicate that, in some manner, the C-terminal portion of DcR3 influences its interaction with these ligands. The specificity of the cleavage suggests that the secondary and tertiary structures of the polypeptide may be involved in the presentation of this cleavage site to the proper protease. These observations make it enticing to speculate that limited proteolysis of endogenous DcR3 could serve as a mechanism regulating its activities under specific pathophysiological conditions. For example, neutrophil-derived serine proteases have been suggested to be involved in the regulation of the expression of functional interleukin-2 and interleukin-6 receptors at focal sites of inflammation [23]. It has been speculated that DcR3 acts as a decoy receptor involved in immune evasion, and may be involved in activated T-cell trafficking and endothelial cell survival [5,6]. So while the *in vivo* functions of DcR3 are not entirely clear, it is conceivable that the expression of DcR3 would be co-regulated along with that of FasL and Fas in order to coordinate apoptotic, immune, and inflammatory responses [8]. Additionally, it is not clear whether or not DcR3 itself may be directly involved in signal transduction through FasL and LIGHT. As such, specific proteolytic conversion of DcR3 to DcR3(1-218) may provide a unique mechanism influencing the balance between the immune response and apoptosis under a variety of circumstances.

Targeted modification of a single amino acid residue has been an approach successfully applied to generate stable peptide analogs of growth hormone-releasing hormone and glucagon-like intestinal peptide, whose biological and functional half-lives are affected negatively after cleavage by dipeptidyl aminopeptidase [24,25]. While engineering these and other small peptides for stability can be directed more easily, successful identification of a single amino acid important to the *in vivo* metabolic stability of a large glycoprotein such as DcR3 is not intuitively obvious. Fundamental to the current approach was the utility of the *in vitro* tissue systems (subcutaneous homogenate, whole blood) in corroborating the degradation pattern of DcR3 observed *in vivo* [26]. The observation that position R218 was also selectively cleaved by thrombin verified the unique sensitivity of this site to proteolysis and provided a simple way to test the effect of the R218Q mutation on metabolic stability. This type of approach has been used previously to successfully develop pepsin-resistant analogs of long-R3-IGF-I, which were more stable to the local environment when administered orally [27].

The biological activity of DcR3 administered extravascularly (i.e. subcutaneously) as a therapeutic protein will be affected by the extent of cleavage at position 218 at the site of administration. Proteolysis at position R218 would

be considered detrimental to the pharmacological potency of DcR3 if efficacy required the interruption of the interaction of both FasL and LIGHT with their cognate receptors or relied upon inhibition of events elicited specifically by FasL. Whether thrombin itself or some other serine protease is responsible for the specific proteolysis of DcR3 observed *in vivo* and in *in vitro* tissue systems is not clear. Although we have not been able to clearly demonstrate the specific proteolysis of DcR3 after intravenous administration to normal animals, cleavage and inactivation of DcR3 administered by this route could occur via the expression/release of proteases in conditions of unregulated inflammatory response [28]. The rational for the single point mutation, R218Q, was to make this site resistant to metabolism by the serine protease responsible for this cleavage *in vivo*. Thus, FLINT was designed to have improved metabolic stability and bioavailability while retaining activities against both FasL and LIGHT. The results of our studies indicated that FLINT was not degraded *in vitro* and was stable to proteolytic degradation by thrombin. Importantly, FLINT showed metabolic stability *in vivo* after subcutaneous administration in both monkeys and mice. Biologically, FLINT was indistinguishable from DcR3 in its binding to sFasL and LIGHT and was as effective in inhibiting sFasL-mediated apoptosis and sLIGHT-mediated inhibition of HT-29 cell proliferation *in vitro*. Therefore, the improved metabolic stability of the analog could translate to a requirement for a lower subcutaneous dose in order to obtain a desired effect. Interestingly, in a murine model of acute, fulminant hepatic apoptosis induced specifically by FasL, intravenous administration of a TR6 (DcR3) Fc fusion protein provided survival benefit to the animals. However, none of the animals survived when DcR3-Fc was administered by the subcutaneous route at a 4-fold higher dose [29]. It is likely that the lack of subcutaneous efficacy observed in this model was due to the proteolysis of DcR3 as described in this report, and is consistent with our observation that the proteolytic fragment does not bind FasL or inhibit FasL-mediated apoptosis.

In conclusion, the findings in this report indicate that DcR3 is proteolytically processed *in vitro* and *in vivo* to DcR3(1-218), which has a differential function in inhibiting FasL- and LIGHT-mediated activities. The modification in the sequence of DcR3 to produce FLINT (LY498919) should result in a pharmacologically superior molecule in the therapeutic intervention of diseases in which the pathogenesis is linked to FasL-mediated apoptotic or inflammatory events.

Acknowledgments

The authors would like to thank Don McClure for expression of DcR3; Joe Manetta for production of antibodies; Larry Mann, Lisa Green, and Phil Marder for performing FACS analysis; Gene Seno, Laura Myers, Bruce Glover, Chris Mundy, Chad Geringer, Jeff Arnold, Wendy Fuller, Joe Berry, Christopher Frye, and Sheng-Hung Tscang for their molecular biology efforts; and Paul Atkinson and Yu Tian for purification of DcR3, FLINT, and metabolite. The authors would also like to thank Songqing Na, Joe Heuer, and Alexei Kharitonenkov for helpful discussions and Joe Jakubowski and Andy Glasebrook for critical review of this manuscript.

References

[1] Armitage RJ. Tumor necrosis factor receptor superfamily members and their ligands. Curr Opin Immunol 1994;6:407–13.
[2] Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell 1994;75:959–62.
[3] Torcia M, Bracci-Laudiero L, Lubicello M, Nencioni L, Labardi D, Rubartelli A, Cozzolino F, Aloi L, Garaci E. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. Cell 1996;85:345–56.
[4] Kramer PH, Behrmann I, Dhein J, Debatin KM. Regulation of apoptosis in the immune system. Curr Opin Immunol 1994;6:279–89.
[5] Yu K-Y, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. J Biol Chem 1999;274:13733–6.
[6] Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, Feng P, Qi S, Chen H, Cho YH, Li Y, Moore PA, Wu J. Modulation of T-cell responses to alloantigens by TR6/DcR3. J Clin Invest 2001;107:1459–68.
[7] O'Connell J, Houston A, Bennett MW, O'Sullivan GC, Shanahan F. Immune priviledge or inflammation? Insights into the Fas ligand enigma. Nature Med 2001;3:271–4.
[8] Maggi CA. Therapeutic opportunities from the pharmacological manipulation of the Fas system. Pharmacol Res 1998;38:1–34.
[9] Patel T, Roberts LR, Jones BA, Gores GJ. Dysregulation of apoptosis as a mechanism of liver disease: an overview. Semin Liver Dis 1998;18:105–14.
[10] Kwon BS, Tan KB, Ni J, Oh KO, Lee ZH, Kim KK, Kim Y-J, Wang S, Gentz R, Yu G-L, Harrop J, Lyn SD, Silverman C, Porter TG, Truneh A, Young PR. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. J Biol Chem 1997;272:14272–6.
[11] Tamada K, Shimozaki K, Chapoval AI, Zhai Y, Su J, Chen S-F, Hsieh S-L, Nagata S, Ni J, Chen L. LIGHT, a TNF-like molecule costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. J Immunol 2000;164:4105–10.
[12] Migone T-S, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen S-F, Zhou JXH, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. Immunity 2002;16:479–92.
[13] Gerlitz B, Hassell T, Vlahos CJ, Parkinson JF, Bang NU, Grinnell BW. Identification of the predominant glycosaminoglycan-attachment site in soluble recombinant human thrombomodulin: potential regulation of functionality by glycosyltransferase competition for serine[474]. Biochem J 1993;295:131–40.
[14] Berg DT, McClure DB, Grinnell BW. High-level expression of secreted proteins from cells adapted to serum-free suspension culture. Biotechniques 1993;14:972–8.
[15] Berg DT, McClure DB, Grinnell BW. E1a-responsive mammalian host/vector system for the stable high-level expression of secreted proteins. Nucleic Acids Res 1992;20:5485–6.

[16] Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996;173:33–8.
[17] Morel Y, Schiano de Colella J-M, Harrop J, Deen KC, Holmes SD, Wattam TA, Khandekar SS, Truneh A, Sweet RW, Gastaut J-A, Olive D, Costello RT. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J Immunol 2000;165: 4397–404.
[18] Korc M, Finman JE. Attenuated processing of epidermal growth factor in the face of marked degradation of transforming growth factor-α. J Biol Chem 1989;264:14990–9.
[19] Powers CA, Hatala MA. Prolactin proteolysis by glandular kallikrein: *in vitro* reaction requirements and cleavage sites and detection of processed prolactin *in vivo*. Endocrinology 1990;127:1916–27.
[20] Cook AL, Kirwin PM, Craig S, Bawden LJ, Green DR, Price MJ, Richardson SJ, Fallon A, Drummond AH, Edwards RM, Clements JM. Purification and analysis of proteinase-resistant mutants of recombinant platelet-derived growth factor-BB exhibiting improved biological activity. Biochem J 1992;281:57–65.
[21] Baumann G. Growth hormone heterogeneity: genes, isohormones, variants and binding proteins. Endocr Rev 1991;12:424–49.
[22] Scharpe S, DeMeester I. Peptide truncation by dipeptidyl peptidase IV: a new pathway for drug discovery? Verh K Acad Geneeskd Belg 2001;63:5–32.
[23] Bank U, Reinhold D, Scheemilch C, Kunz D, Synowitz HJ, Ansorge S. Selective proteolytic cleavage of IL-2 receptor and IL-6 receptor ligand binding chains by neutrophil-derived serine proteases at foci of inflammation. J Interferon Cytokine Res 1999;19:1277–87.
[24] Frohman LA, Downs TR, Heimer EP, Felix AM. Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma. J Clin Invest 1989;83:1533–40.
[25] Burcelin R, Dolci W, Thorens B. Long-lasting antidiabetic effect of a dipeptidyl peptidase IV-resistant analog of glucagon-like peptide-1. Metabolism 1999;48:252–8.
[26] Wroblewski VJ, Masnyk M, Becker GW. Proteolytic cleavage of human growth hormone (hGH) by rat tissues *in vitro*: influence on the kinetics of exogenously administered hGH. Endocrinology 1991;129:465–74.
[27] Bryant KJ, Read LC, Forsberg G, Wallace JC. Design and characterisation of long-R3-insulin-like growth factor-I muteins which show resistance to pepsin degradation. Growth Factors 1996;13:261–72.
[28] Dhainaut JF, Marin N, Mignon A, Vinsonneau C. Hepatic response to sepsis: interaction between coagulation and inflammatory processes. Crit Care Med 2001;29:S42–7.
[29] Connolly K, Cho YH, Duan R, Fikes J, Gregorio T, Lafleur DW, Okoye Z, Salcedo TW, Santiago G, Ullrich S, Wei P, Windle K, Wong E, Yao X-T, Zhang Y-Q, Zheng G, Moore PA. In vivo inhibition of Fas ligand-mediated killing by TR6, a Fas ligand decoy receptor. J Pharmacol Exp Ther 2001;298:25–33.