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Cathepsin X cleavage of the β_2 integrin regulates talin-binding and LFA-1 affinity in T cells

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ABSTRACT

T cell migration, essential for immune surveillance and response, is mediated by the integrin LFA-1. CatX, a cysteine carboxypeptidase, is involved in the regulation of T cell migration by interaction with LFA-1. We show that sequential cleavage of C-terminal amino acids from the β_2 cytoplasmic tail of LFA-1, by CatX, enhances binding of the adaptor protein talin to LFA-1 and triggers formation of the latter's high-affinity form. As shown by SPR analysis of peptides constituting the truncated β_2 tail, the cleavage of three C-terminal amino acids by CatX resulted in a 1.6-fold increase of talin binding. Removal of one more amino acid resulted in a 2.5-fold increase over the intact tail. CatX cleavage increased talin-binding affinity to the MD but not the MP talin-binding site on the β_2 tail. This was shown by molecular modeling of the β_2 tail/talin F3 complex to be a result of conformational changes affecting primarily the distal-binding site. Analysis of LFA-1 by conformation-specific mAb showed that CatX modulates LFA-1 affinity, promoting formation of high-affinity from intermediate-affinity LFA-1 but not the initial activation of LFA-1 from a bent to extended form. CatX post-translational modifications may thus represent a mechanism of LFA-1 fine-tuning that enables the trafficking of T cells. ***J. Leukoc. Biol.*** **90: 99–109; 2011.**

Introduction

Integrins are cell surface adhesion receptors that mediate cell–cell and cell–ECM interactions. They are heterodimers of α and β subunits, each containing a large extracellular domain (80–150 kDa), a single transmembrane α-helix, and a short, largely unstructured cytoplasmic domain or "tail" [1]. The cytoplasmic tails of β integrins comprise between 40 and 60 aa and possess no enzymatic or actin-binding activity of their own. Instead, various adaptor proteins bind to specific sites on integrin tails to mediate integrin activation and clustering and to serve as nucleation points for the assembly of larger signaling and structural scaffolds. At least 42 proteins have been identified that reportedly bind just to the cytoplasmic tails of β integrins [2]. Among these proteins, the cytoskeletal protein talin has been shown to play a pivotal role in integrin-mediated events [3].

Talin 1 (referred to here as talin) promotes integrin clustering [4] and the switching of integrins from inactive to ligand-binding states [5, 6]. It contains an N-terminal head domain (45 kDa), which includes the principal integrin-binding site, and a large C-terminal rod domain (190 kDa), which contains lateral integrin-binding sites and binding sites for actin and vinculin. The talin head domain resembles a well-defined band 4.1, ezrin, radixin, and moesin domain with three subdomains—F1, F2, and F3 [7]. The F3 subdomain contains a PTB site, which binds to the NPXY sequence motif present in β integrin cytoplasmic tails and is involved in integrin activation [8]. β_3 integrin activation has been proposed to be a two-step process, requiring the initial binding of talin F3 to the high-affinity binding site in the MD region of the β_3 integrin tail and a subsequent step in which talin F3 engages the lower-affinity, MP-binding site [7], leading to the separation of the α and β cytoplasmic tails and the transition from bent to extended form [9].

Abbreviations: CatX/L=cathepsin X/L, HEK=human embryonic kidney, his=histidine, MD=membrane distal, MDm=mutation of the membrane distal talin-binding site, MDm-4=mutation of the membrane distal talin-binding site-last 4 aa cleaved by cathepsin X, MP=membrane proximal, MPm=mutation of the membrane proximal talin-binding site, MPm-4=mutation of the membrane proximal talin-binding site-last 4 aa cleaved by cathepsin X, nc=negative control, PTB=phosphotyrosine-binding, RNAi=RNA interference, RU=resonance unit(s), SDF-1=stromal cell-derived factor 1, siRNA=small interfering RNA, SPR=surface plasmon resonance, wt-3/4=WT-last 3/4 aa cleaved by cathepsin X

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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The binding of talin and other adaptor proteins to the β integrin cytoplasmic tail has to be controlled temporally and spatially. Possible mechanisms involve the phosphorylation of residues of the β integrin cytoplasmic tail [10] and proteolytic cleavage. The cysteine proteases calpain and CatX are the two enzymes known to be capable of cleaving the β integrin cytoplasmic tail. Calpains can cleave integrin β_{1A}, β_{1D}, β_2, β_3, and β_7 tails between and adjacent to their NPXY/NXXY motifs [11]. CatX, a carboxypeptidase, abundantly expressed in cells of the immune system [12], has been identified as another candidate for β cytoplasmic tail cleavage. In our recent studies, we demonstrated the colocalization of CatX with LFA-1 (integrin $\alpha_L\beta_2$) in T cells and gradual cleavage of the C terminus of the LFA-1 β_2 cytoplasmic tail, regulating the binding of another adaptor protein, α-actinin-1. Additionally, the cleavage of LFA-1 by CatX was shown to regulate LFA-1 affinity, an event that controls T cell migration and morphology [13, 14].

LFA-1 is expressed exclusively in leukocytes and is of fundamental importance to the function of the immune system. LFA-1 mediates cell adhesion to ICAMs on other leukocytes and endothelial cells under various conditions, e.g., during leukocyte emigration from the bloodstream into tissues and during immunological synapse formation between the T cell and the APC [15]. Talin is implicated in the regulation of LFA-1 function. Overexpression of the talin head domain induces separation of α and β cytoplasmic tails and enhances adhesiveness to ICAM-1 [16]. Activation of β_2 integrin is referred to as the transition from bent, inactive to extended, ligand-binding conformation. Active LFA-1 possesses a closed, extended conformation that binds ICAM-1 with intermediate affinity or an open, extended conformation that binds ICAM-1 with high affinity [17]. The transition between intermediate- and high-affinity LFA-1 will be referred to as LFA-1 affinity modulation.

In this study, we investigated the effects of CatX cleavage of the β_2 integrin cytoplasmic tail on talin-binding affinity. We showed that truncation of the C-terminal of the β_2 tail promotes talin binding by altering the conformation of the MD but not the activation-associated, MP-binding region. Accordingly, this post-translational modification increases LFA-1 affinity for ICAM-1 without affecting its activation.

MATERIALS AND METHODS

Materials

The peptides β_2 wt, β_2 wt-3, β_2 MPm, and β_2 MDm (**Table 1**) were synthesized by Biosynthesis (Denver, TX, USA). The peptides β_2 wt, β_2 MPm, and β_2 MDm were digested with rCatX (0.4 mM) for 1 h at 37°C in 50 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM DTT (pH 5.5) to yield the four-residue shorter peptides β_2 wt-4, β_2 MPm-4, and β_2 MDm-4. The samples were separated by reverse-phase HPLC using a Gemini C18 column (5 μm, 110 Å, 150×46 mm; Phenomenex, Torrance, CA, USA), and the peak fractions were analyzed by Q-TOF Premier mass spectrometer (in electrospray ionization+ mode; Waters, Milford, MA, USA) to confirm the sequences [18].

Antibodies against β_2 integrin were goat anti-human integrin LFA-1 N-18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse mAb KIM127 (American Type Culture Collection, Manassas, VA, USA; CRL-2838), mouse mAb MEM-148, and mAb 24-PE (Santa Cruz Biotechnology). Antibodies for talin were goat anti-human talin C-20 (Santa Cruz Biotechnology) and anti-6× His-tag antibodies (Abcam, Cambridge, MA, USA) for talin head His-tag. Fluorescent antibody conjugates for flow cytometry were prepared by means of the Zenon mouse IgG labeling kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's protocols.

CLIK-148, a specific inhibitor of CatL [19], was provided by Prof. Nobuhiko Katunuma (Tokushima Bunri University, Japan). CatX-specific epoxysuccinyl-based inhibitor AMS36 was provided by Prof. Mathew Bogyo (Stanford University, Palo Alto, CA, USA). Neutralizing mAb against CatX (2F12 mAb) was obtained from a mouse hybridoma cell line [12].

TABLE 1. Peptides Representing β_2 Integrin Cytoplasmic Tail Used in Experiments

Peptide	Name	Amino acid (aa) sequence	Description
β_2 (wt)	β_2 wt	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES	WT
β_2 (Δ767–769)	β_2 wt-3	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKF	Last 3 aa cleaved by CatX.
β_2 (Δ766–769)	β_2 wt-4	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPK	Last 4 aa cleaved by CatX.
β_2 (W747A/F754A)	β_2 MDm	KALIHLSDLREYRRFEKEKLKSQANNDNPLAKSATTTVMNPKFAES	Mutation of the membrane distal talin-binding site.
β_2 (W747A/F754A/ Δ766–769)	β_2 MDm-4	KALIHLSDLREYRRFEKEKLKSQANNDNPLAKSATTTVMNPK	Mutation of the membrane distal talin-binding site and last 4 aa cleaved by CatX.
β_2 (Y735A/F738A/ Δ766–769)	β_2 MPm	KALIHLSDLREARRAEKEKLKSQWNNDNPLFKSATTTVMNPKFAES	Mutation of the membrane proximal talin-binding site.
β_2 (Y735A/F738A)	β_2 MPm-4	KALIHLSDLREARRAEKEKLKSQWNNDNPLFKSATTTVMNPK	Mutation of the membrane proximal talin-binding site and last 4 aa cleaved by CatX.
β_2 (Y735A/F738A/ W747A/F754A)	β_2 nc	KALIHLSDLREARRAEKEKLKSQANNDNPLAKSATTTVMNPKFAES	Mutation of the membrane distal and proximal talin-binding sites (negative control).

Expression constructs

The α_L and β_2 expression constructs were from Addgene (Cambridge, MA, USA; Plasmids 8630 and 8640, respectively). The truncated forms β_2 wt-3 (Δ767–769) and β_2 wt-4 (Δ766–769) of the β_2 integrin subunit were prepared from Plasmid 8640 with the PCR. Primers (Eurofins, Denmark) used for PCR multiplication were: forward-TAAACTTAAGCTTCTAGAGATC-CCTC, reverse (β_2 wt-3)-TTATGATATCTCAAAACTTGGGGTTCATG, and reverse (β_2 wt-4)-TTATGATATCTCACTTGGGGTTCATGAC. The PCR products were cut with *HindIII* and *EcoRV* restriction endonucleases and ligated into pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA, USA). Concentration and quality of the plasmid samples were controlled spectrophotometrically (NanoDrop, Thermo Fisher Scientific, Wilmington, DE, USA), and their correct sequences were confirmed by sequencing (Eurofins). The talin head cDNA (1–439 aa), fused to an N-terminal His6-tag in pET23a plasmid, was provided by Dr. Mirko Himmel (Interdisciplinary Centre for Clinical Research, Leipzig, Germany). His-tagged talin head was expressed in the *Escherichia coli* strain BL21(DE3) and purified on Ni-NTA agarose (Qiagen, Valencia, CA, USA), according to the manufacturer's protocols.

Cells and transfection

Jurkat T cell line (TIB-152) was cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. CatX-overexpressing Jurkat T cells were prepared and analyzed for CatX expression as described [13]. HEK293T cells were cultured in DMEM (Sigma-Aldrich), supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. HEK293T cells were transfected using the calcium phosphate method. Buffy coats from the venous blood of normal, healthy volunteers were obtained from the Blood Transfusion Centre of Slovenia, according to institutional guidelines. PBMCs were isolated using Lympholyte-H (Cedarlane Laboratories, Ontario, Canada). Whole $CD4^+$ T cells were obtained by positive selection using CD4 microbeads (Milteny Biotec GmbH, Bergisch Gladbach, Germany), following the manufacturer's protocol. The purity of $CD4^+$ T cells was always >95%, as determined by flow cytometry. Jurkat and $CD4^+$ T cells were transiently transfected with control and CatX-specific siRNA (CTSZHSS102509; Invitrogen) using Lipofectamine 2000 (Invitrogen).

SDS-PAGE and Western blot

Jurkat T cells were incubated overnight in 5 μM AMS36 and 1 μM 2F12 or left untreated. Cells were lysed in ice-cold HEPES-NaCl lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, and 1% Triton X-100), complemented with inhibitors of proteases [0.2 mg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride, 2 μg/mL aprotinin, and 10 μg/mL leupeptin] and phosphatases (50 mM NaF, 10 mM $Na_4P_2O_7$, and 10 mM $NaVO_4$). Postnuclear cell lysates were heated at 100°C with reducing SDS sample buffer and analyzed by high-resolution SDS-PAGE. For optimal resolution, samples were analyzed using Anderson gels as described [20]. Primary antibodies used for Western blotting were goat anti-human integrin LFA-1 N-18 recognizing the N terminus of human β_2 integrin.

Immunoprecipitation

Jurkat T cells were transiently transfected with control and CatX-specific siRNA and incubated overnight. CatX-overexpressing Jurkat T cells were incubated overnight with AMS36 at a final concentration of 2.5 μM. The cells (3.5×10^7) were then incubated with 20 μg/ml KIM127 in RPMI 1640 (Sigma-Aldrich), supplemented with 0.5% FCS and 20 mM HEPES for 30 min at 37°C, and then washed to remove unbound antibody. The cells were lysed with ice-cold HEPES-NaCl lysis buffer complemented with inhibitors. The T cell lysate was incubated with 50% v/v of Sepharose-linked proteins G and A (Amersham Biosciences, Piscatway, NJ, USA) for 4 h at 4°C and then washed twice with lysis buffer before SDS-PAGE separation. Diluted T cell lysates (10×) were used as a positive control. Nitrocellulose blots were probed with goat anti-human talin C-20.

Immunofluorescence microscopy

Freshly isolated $CD4^+$ T cells and Jurkat cells were cytospinned or seeded on ICAM-1-Fc-precoated slides and allowed to migrate for 30 min. The migrating cells were fixed for 45 min by direct addition of 4% paraformaldehyde in PBS without removing the medium. They were permeabilized by 0.1% Triton X-100 in PBS for 10 min and nonspecific staining blocked with 3% BSA in PBS for 1 h. CatX was labeled with 2F12 mAb (10 μg/mL) and talin with goat anti-human talin (5 μg/mL; Santa Cruz Biotechnology). After 2 h at room temperature, the cells were washed with PBS and treated with Alexa-labeled secondary antibodies (Molecular Probes) for 2 h. After washing with PBS, ProLong antifade kit (Molecular Probes) was mounted on dried slides and allowed to dry overnight at room temperature. Fluorescence microscopy was performed using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss LSM Image Software 3.0).

SPR

The real-time binding of the talin head domain to β_2 integrin cytoplasmic tail peptides β_2 wt, β_2 wt-3, β_2 wt-4, β_2 MPm, β_2 MPm-4, β_2 MDm, and β_2 MDm-4 was analyzed by SPR using the Biacore X (Biacore, Piscataway, NJ, USA). Anti-6× His-tag antibody was immobilized at the carboxymethyldextran surface of a CM5 chip (Biacore) using amine coupling, following the manufacturer's instructions. Anti-6× His-tag antibody was diluted to a final concentration of 30 μg/mL in acetate buffer (pH 5.5) and immobilized on the surface at a flow rate of 5 μL/min for 7 min. His-tagged talin head domain was diluted in running buffer (HBS-EP; pH 7.4) at a final concentration of 50 μg/mL and bound to immobilized anti-6× His-tag antibody at a flow rate of 5 μL/min for 5 min. For kinetic measurements, different concentrations of β_2 integrin cytoplasmic tail peptides in running buffer were injected for 5 min at a flow rate of 5 μL/min, followed by a 2-min dissociation phase. At the end of each cycle, the surface was regenerated with 0.01M glycine (pH 9.5). The equilibrium rate constants (K_d values) were calculated using a Langmuir model (1:1 binding with drifting baseline) with the Biacore evaluation software BIAevaluation, Version 3.2 (Biacore AB, Chalfont St. Giles, UK).

Molecular modeling

3D structures of peptides (β_2 wt, β_2 wt-3, β_2 wt-4, and β_2 nc; Table 1) with talin were prepared with Sybyl 8.0 (Tripos, St. Louis, MO, USA). The crystal structure of talin F3 bound to the β_3 integrin cytoplasmic tail peptide [9] (Protein Data Bank Entry 2H7D) was used as a template. Amino acids from the β_3 integrin cytoplasmic tail peptide were replaced with β_2 integrin cytoplasmic tail peptide sequences. Minimization was performed by NAMD [21] with graphic user interface Visual Molecular Dynamics [22]. Each complex β_2 (wt, wt-3, wt-4, nc)/talin F3 was modeled in a solvation box with water and ions. Each complex was then minimized for 10,000 steps. After minimization, the energy of the interaction between each β_2 peptide and talin F3 was calculated. A molecular dynamics simulation (10,000 steps) was run for each complex to confirm the stability of the system.

Flow cytometry analysis

Freshly isolated $CD4^+$ T cells, transfected HEK293T cells, wt, and CatX-overexpressing Jurkat T cells were incubated overnight in 5 μM AMS36 and 3 μM CLIK-148. The chemokine SDF-1 (R&D Systems, Minneapolis, MN, USA; 1 μg/mL) was added for 1 h where indicated. Freshly isolated $CD4^+$ T cells were transiently transfected with control and CatX-specific siRNA and incubated overnight. Formalin (500 μl; 10%) was added directly to 50 μl cells, which were fixed for 30 min at 37°C with 5% CO_2. Thereafter, the cells were incubated by prelabeled antibodies N-18 (β_2), KIM127, MEM-148, and mAb 24 for 30 min at 4°C. The cells were then washed with PBS and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Diego, CA, USA).

Cell spreading

Freshly isolated $CD4^+$ T cells were transiently transfected with control and CatX-specific siRNA and incubated overnight. The wells of an eight-well LabTek chambered coverglass system (Nalge Nunc International, Rochester, NY, USA) were coated overnight with 5 μg/mL ICAM-1 (R&D Systems) together with 2 μg/mL SDF-1. Cell suspension (200 mL; 10^5 cells/mL) was added to the wells and allowed to spread for 24 h at 37°C with 5% CO_2. Images were taken by an Olympus IX81 motorized, inverted microscope and CellR software. At least 300 individual cells were analyzed for the spreading phenotype/condition in two separated experiments.

Statistical analysis

SPSS personal computer software (Release 13.0) was used for statistical analysis of all data. Differences between the groups were evaluated using the nonparametric Mann-Whitney test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

CatX induces truncated β_2 integrin in T cells

CatX cleaves the β_2 integrin cytoplasmic tail gradually after the C-terminal amino acids S769, E768, A767, and F766 [18, 23]. These results were obtained in vitro using a synthetic peptide constituting the entire β_2 cytoplasmic tail. To confirm the proposed post-translational modification of β_2 integrin by CatX at the cellular level, we analyzed extracts of wt and CatX-overexpressing Jurkat cells. High-resolution SDS-PAGE analysis identified a faster migrating β_2 integrin form in the extracts of CatX-overexpressing Jurkat cells (**Fig. 1A**). As CatX has been shown to remove a maximum of 4 aa, including a negatively charged glutamate residue (E768), from the C-terminal of β_2 integrins, the smaller β_2 integrin form is presumed to be a truncated β_2 integrin (Δ766–769). In contrast, wt Jurkat cells expressed significantly lower amounts of the truncated β_2 integrin form. Importantly, the expression of β_2 integrin on the cell surface was identical for wt and CatX-overexpressing Jurkat cells, as determined by flow cytometry (see Supplemental Material 2). Moreover, generation of the truncated β_2 integrin form was reduced significantly when CatX-overexpressing Jurkat cells were pretreated with specific CatX inhibitors 2F12 and AMS36. The overexpression of CatX in CatX-overexpressing Jurkat cells was confirmed by specific ELISA [13]. The levels of CatX were 3.5-fold higher compared with wt Jurkat cells (Fig. 1B).

Truncation of the β_2 integrin cytoplasmic tail affects its affinity for talin

The effect of cleaving the β_2 integrin C-terminal residues on β_2 integrin affinity for talin was evaluated by SPR, using the β_2 wt-3 and β_2 wt-4 peptides as models for the 3- and 4-aa shorter β_2 integrin subunits produced by CatX. The talin head domain was coupled to the flow cell of the Biacore sensor chip CM5, and various concentrations of the β_2 wt, β_2 wt-3, and β_2 wt-4 peptides (Table 1) were added to the flow cell. The interaction of these soluble cytoplasmic tail peptides with the immobilized talin head domain was measured in arbitrary units (RU), and affinity changes were calculated from the maximal RU responses. The binding affinities of β_2 wt-3 and β_2 wt-4 peptides were, respectively, 1.6- and 2.5-fold higher than that of the β_2 wt peptide (**Fig. 2A** and **B**).

To reveal the structural basis of the modified β_2 integrin cytoplasmic tail/talin interaction, we constructed a model using the atomic coordinates of the talin F3 domain when bound to the β_3 integrin cytoplasmic tail peptide [9] as a template. The β_3 and β_2 integrin cytoplasmic tails possess considerable sequence similarity, and the heterogeneous amino acids have been substituted to obtain a β_2 tail/talin F3 complex. Talin F3 was bound in silico to four peptides—β_2 wt, β_2 wt-3, β_2 wt-4, and β_2 nc. The interaction energies were calculated after 10,000 steps of minimization for each peptide. The calculated interaction energies were in accord with the SPR results, the highest for β2 wt-4 and the lowest for β_2 wt. The interaction energies determined for the MD talin-binding region WNNDNPLFKS [7, 9] of β_2 wt and of β_2 wt-4 differed significantly (**Table 2** and **Fig. 3A**). Visual comparison of complexes

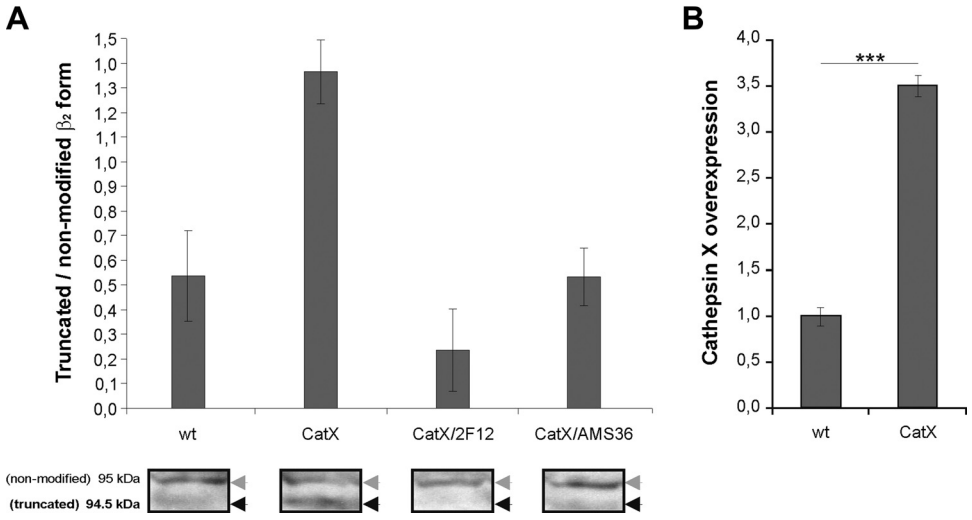

Figure 1. Analysis of the presence of the truncated β_2 integrin form in Jurkat T cells by high-resolution SDS-PAGE. (A) Cell extracts of wt Jurkat cells (wt), CatX-overexpressing Jurkat cells (CatX), and CatX-overexpressing Jurkat cells pretreated with 2F12 (CatX/2F12) or AMS36 (CatX/AMS36) were separated by high-resolution SDS-PAGE followed by Western blotting with β_2 integrin-specific antibodies. The presence of the truncated β_2 form can be observed (black arrowheads) under the nonmodified, 95-kDa form (white arrowheads). The intensity of the β_2 forms was quantified by densitometry and is presented as the ratio between the truncated versus the nonmodified β_2 form. Values are the mean ± SD of two separate experiments. (B) Cell extracts of wt Jurkat cells and CatX-overexpressing Jurkat cells were analyzed for CatX expression by specific ELISA. Values are means ± SD and result from three separate experiments (***$P<0.001$).

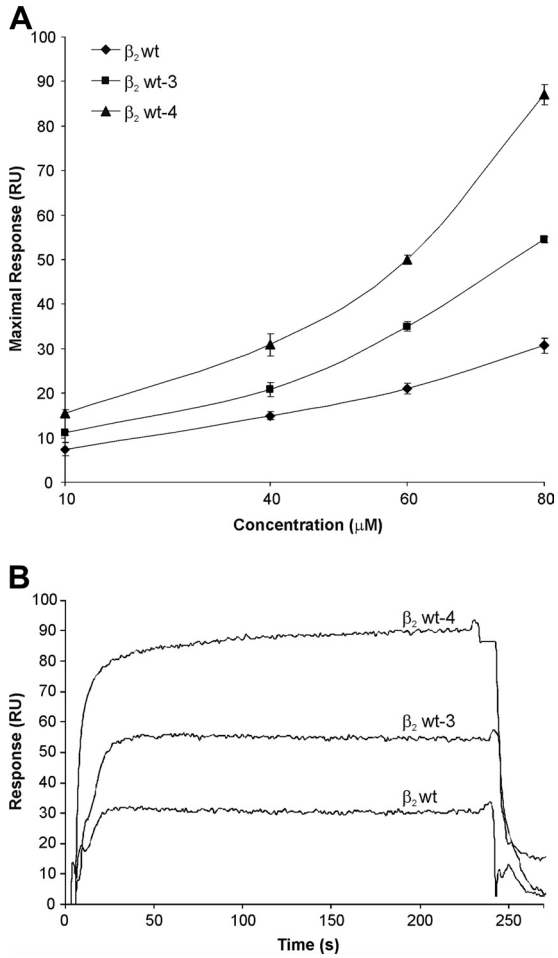

Figure 2. SPR analysis of talin head domain interaction with β_2 cytoplasmic tail peptides. (A) The interaction of β_2 wt, β_2 wt-3, and β_2 wt-4 peptides with the immobilized talin head domain was measured by SPR (Biacore). Maximal RU are shown as a function of peptide concentration (10, 40, 60, and 80 μM). Values are mean ± SD. (B) SPR sensograms of the talin head domain interaction with β_2 wt, β_2 wt-3, and β_2 wt-4 at 80 μM concentration.

β_2 wt/talin F3 and β_2 wt-4/talin F3 shows no drastic structural perturbations (Fig. 3B). The conformation of the NPLF motif in the MD talin-binding region, which has been shown to associate with the PTB domain of talin F3 and to be crucial for formation of the integrin/talin complexes [6, 24], was not altered by removal of the four C-terminal amino acids from the β_2 tail. However, the positions of the residues N749 and D750 (–2 and –1, with respect to the NPLF motif) differed significantly. In β_2 wt-4, they appear to make more contacts with talin F3 than in β_2 wt. The Cα and Cγ positions of N749 differ by 1.6Å and 2.6Å and of D750 by 2Å and 3.7Å (Fig. 3C). Collectively, these results suggest that CatX cleavage of the β_2 integrin cytoplasmic tail increases the affinity for talin by altering the conformation of the MD talin-binding region.

TABLE 2. Interaction Energies between Talin F3 and β_2 Integrin Cytoplasmic Tail Peptides

	Electrostatic (kcal/mol)	Van der Walls (kcal/mol)	Total (kcal/mol)
β_2 nc	–360	–43	–403
β_2 wt	–352	–79	–432
β_2 wt-3	–370	–84	–455
β_2 wt-4	–376	–83	–459
WNNDNPLFKS (wt)	–14	–40	–54
WNNDNPLFKS (–4)	–26	–39	–65

Inhibition of CatX reduces the association of talin with the β_2 subunit in T cells

Coimmunoprecipitation of talin with LFA-1 by mAb KIM127 revealed that it is bound to the β_2 integrin subunit of wt and CatX-overexpressing Jurkat cells. Preincubation of CatX-overexpressing Jurkat cells with CatX inhibitor AMS36 and RNAi silencing of CatX in wt Jurkat cells reduced the coimmunoprecipitation of talin with LFA-1, implying that β_2 tail cleavage by CatX enhances β_2 integrin affinity for talin (**Fig. 4A**). The RNAi silencing of CatX in wt Jurkat cells was 25%, as confirmed by ELISA (Supplemental Material 1). To provide additional evidence that CatX has an impact on the interaction of active LFA-1 and talin, detailed fluorescence microscopy analysis (≥30 cells) of their colocalization was performed (Fig. 4B). The colocalization of immunolabeled talin with KIM127 mAb-positive LFA-1 in CatX-overexpressing cells was more intense than in wt Jurkat cells and occurred along the cell membrane (Fig. 4C). As expected, CatX was also found to be colocalized with talin, suggesting that the affinity-modulating cleavage of the β_2 tail may occur while talin is bound to LFA-1 (Fig. 4D).

CatX is not involved in LFA-1 activation, but it modulates LFA-1 affinity and T cell spreading

Besides direct interference by CatX, CatL, a cysteine endopeptidase, has been proposed to affect LFA-1 affinity indirectly in T cells by activating pro-CatX [25]. Further investigations of the involvement of CatX and CatL in LFA-1 affinity regulation were performed by flow cytometry using reporter mAb KIM127 and MEM-148 to detect extended, active conformations (intermediate- and high-affinity LFA-1; referred to as LFA-1 activation) and mAb 24 to detect exclusively the high-affinity LFA-1 [26, 27]. Freshly isolated $CD4^+$ T cells were pretreated with specific CatX or CatL inhibitors, AMS36 or CLIK148, respectively, and the influence of cathepsin inhibition on LFA-1 activation was measured by mAb KIM127 and MEM-148. The effect of cathepsin inhibitors on LFA-1 activation was not significant for $CD4^+$ T cells (**Fig. 5A** and Supplemental Material 2), suggesting that cleavage of the β_2 tail by CatX per se is not sufficient to trigger LFA-1 activation. In contrast, cathepsin inhibition caused a significant decrease of high-affinity LFA-1 expression in $CD4^+$ T cells, as detected by mAb 24.

As in $CD4^+$ T cells, CatX inhibition did not affect LFA-1 activation in wt or CatX-overexpressing Jurkat cells (Fig. 5B; Supplemental Material 2). The ability of CatX to regulate LFA-1 affinity remains unquestionable in Jurkat cells; however, its ability to promote high-affinity LFA-1 was delayed compared

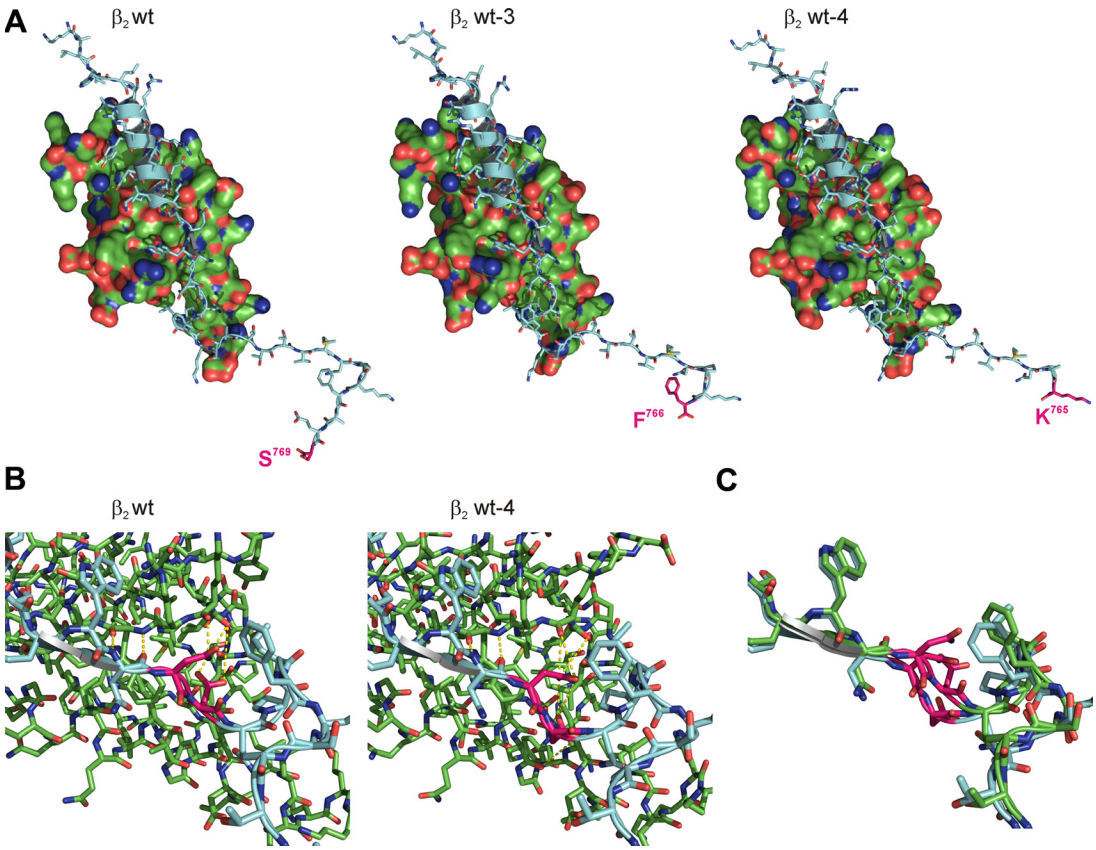

Figure 3. A proposed model of β_2 cytoplasmic tail/talin F3 after CatX processing. (A) Overall structure of β_2 wt, β_2 wt-3, and β_2 wt-4 peptides, shown as a licorice model (cyan), docked to talin F3, shown as a surface representation. The C-terminal amino acid residue prior to (β_2 wt) and after (β_2 wt-3 and β_2 wt-4) processing by CatX is shown in pink. (B) Expanded view of the docked interface of the talin F3 with the MD-binding site of the β_2 cytoplasmic tail. The positions of N749 and D750 (shown in pink) are different for β_2 wt and β_2 wt-4. (C) Alignment of the MD-binding site structure of β_2 wt (green) and β_2 wt-4 (cyan). The images were produced with the program PyMOL (http://www.pymol.org/).

with that in $CD4^+$ T cells. This is in agreement with our previous studies, where CatX-induced, high-affinity LFA-1 required prolonged costimulation by outside-in activating stimuli, such as multivalent ICAM-1, or enclosing the cells in a restrictive ECM [14, 25]. Differences in LFA-1 regulation between primary T cells and Jurkat T cells have been observed before and could be caused by several factors, including variations in the expression of one or more cytoskeletal adaptors [28].

To clarify the association of CatX-modified β_2 integrin subunits with different LFA-1 affinity conformations, we transiently transfected HEK293T cells with α_L and different β_2 expression constructs representing wt (β_2 wt) and truncated β_2 integrin isoforms (β_2 wt-3 and β_2 wt-4). LFA-1 transfection efficiency, evaluated by activation-specific mAb KIM127, was similar for all combinations of transfection constructs, except for the control without the β_2 integrin subunit (Fig. 5C, upper panel; Supplemental Material 2). The lower panel in Fig. 5C indicates the percentage of active LFA-1 (KIM127-positive LFA-1), which is in the high-affinity conformation (mAb 24-positive LFA-1). Cells transfected with β_2 wt-3 expressed significantly lower levels of high-affinity LFA-1 than cells transfected with the wt β_2 integrin subunit, suggesting that most of the active β_2 wt-3 LFA-1 adopted the intermediate-affinity conformation. On the other hand, the levels of high-affinity LFA-1 conformations were significantly higher for β_2 wt-4 transfection or for the cotransfection of the β_2 wt integrin subunit with CatX. Collectively, these results are in accord with our previous studies and confirm that the regulation of LFA-1 affinity by CatX is a complex process, as high-affinity LFA-1 is achieved through an intermediate stage in which LFA-1 adopts the intermediate-affinity conformation.

The results from RNAi-mediated silencing of CatX in $CD4^+$ T cells concord with the results obtained with the pharmacological inhibitor AMS36. CatX silencing in $CD4^+$ T cells did not affect the levels of the total β_2 integrin subunit or active LFA-1, and high-affinity LFA-1 expression was reduced by 26 ± 5% (**Fig. 6A**). The RNAi silencing of CatX in $CD4^+$ T cells was 25% as confirmed by ELISA (Supplemental Material 1). Further, RNAi silencing of CatX limited the ability of $CD4^+$ T cells to spread on a ICAM-1/SDF-1-coated surface (Fig. 6B and C). Although 75 ± 15% of control $CD4^+$ T cells exhibited the spreading phenotype, only 23 ± 7% of CatX-silenced cells were able to spread.

To test the ability of CatX to modulate LFA-1 affinity in the presence of chemokine stimulation, wt Jurkat cells were pretreated with AMS36 and stimulated with the chemokine SDF-1

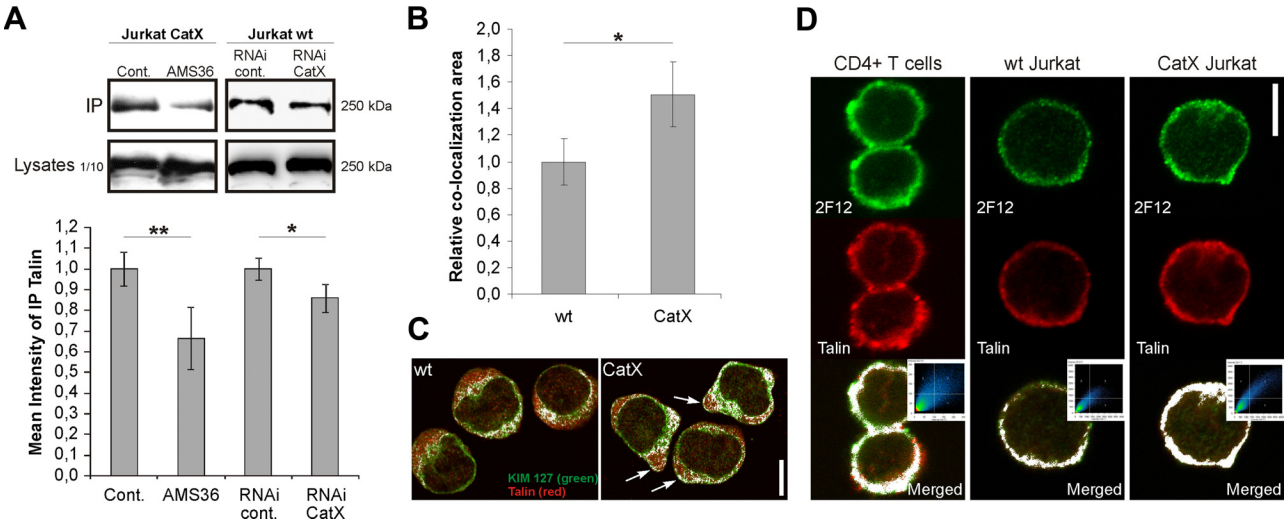

Figure 4 . Coimmunoprecipitation of talin with the β_2 subunit and colocalization with CatX. (A) CatX activity in Jurkat T cells was reduced by AMS36 or RNAi silencing. β_2 subunit-coimmunoprecipitated (IP) talin (235 kDa) was detected by talin-specific antibodies after SDS-PAGE and Western blot. Diluted T cell lysates (10×) were used as controls (Lysates 1/10). The intensities of immunoprecipitated talin were quantified by densitometry and are representative of two independent experiments. Values are means ± SD (*P<0.05; **P<0.01). (B, C) CatX promotes colocalization of LFA-1 (KIM127; green) and talin (red). Fluorescent dyes were imaged sequentially in a frame-interlace mode to eliminate cross-talk between the channels. Colocalization is represented by the pixels above the threshold in both channels. The threshold level corresponds to one-half of the maximal brightness level. The relative colocalization area (white color) was analyzed for several wt and CatX-overexpressing T cells (cell number ≥30). Values are means ± SD (*P<0.05). Perimembrane colocalization of LFA-1 and talin could be observed in CatX-overexpressing T cells (arrows). (D) T cells were allowed to migrate on ICAM-1-precoated slides for 30 min, and colocalization of talin and CatX was analyzed. The threshold level for this display corresponds to one-third of the maximal brightness level. Colocalization is represented by the pixels above the threshold in both channels on the contour plot and on the merged image (white color). Original scale bar, 5 μm.

for 1 h. After SDF-1 stimulation, all active LFA-1 on control wt Jurkat cells adopted the high-affinity conformation (**Fig. 7**). When CatX was inhibited by AMS36, SDF-1 stimulation failed to promote the conversion of active LFA-1 to high-affinity LFA-1.

CatX increases talin-binding affinity for the MD-binding site

Integrin activation by talin is a two-step process in which the talin head domain first recognizes the high-affinity binding site in the MD region of the resting integrin, with subsequent binding to a second, lower-affinity MP site that triggers separation of the α and β integrin tails [7, 9]. To demonstrate that CatX affects talin affinity for the MD and not the MP-binding site of the β_2 integrin, we performed a SPR analysis of talin binding to mutated β_2 integrin cytoplasmic tail peptides (Table 1). Exclusive binding of talin to the MP-binding site was tested on the β_2 MDm peptide, in which point mutations W747A and F754A were introduced in the MD-binding site. These mutated residues are important for talin binding to the MD-binding site [7]. The interactions of the talin head domain with β_2 MDm (K_d 375±75) and β_2 MDm-4 (K_d 322±126 mM) peptides were very weak (**Fig. 8A** and **B**). The MP-binding site promotes integrin activation, and our results demonstrate that CatX does not affect integrin activation. Thus, it was expected that CatX cleavage would have no significant impact on talin binding to the MP-binding site. The opposite approach, i.e., β_2 MPm peptide, with point mutations Y735A and F738A, which prevent binding to the MP-binding site [9], was used to analyze the binding of talin to the high-affinity, MD-binding site. As expected, the interaction between the talin and β_2 MPm peptide (K_d 8.54±1.96 μM) was stronger than that with the β_2 MDm peptide, revealing the importance of the intact β_2 MD-binding site for talin binding. Furthermore, modification of the β_2 MPm peptide to β_2 MPm-4 by CatX caused an additional increase in talin-binding affinity (K_d 3.47±0.64 nM; Fig. 8, A and C). Our molecular models of β_2 peptide/talin F3 and the SPR results together indicate that CatX cleavage at the C terminus of the β_2 tail increases talin affinity for the MD-binding site rather than affecting activation-associated binding to the MP site.

DISCUSSION

A new mechanism of integrin inside-out signaling has been demonstrated. It involves fine regulation of the binding of the adaptor protein talin to β_2 integrin LFA-1 by proteolytic cleavage with CatX, which results in increased affinity of LFA-1 for its counterpart ICAM-1. The major findings are as follows: (1) C-terminal cleavage of the β_2 cytoplasmic tail by CatX progressively promotes its affinity for talin; (2) CatX controls the interaction of talin with the MD but not the activation-associated MP region of the β_2 tail; (3) CatX cleavage of the β_2 tail affects LFA-1 affinity but not LFA-1 activation in T cells.

The LFA-1 integrin acts at several levels of the T cell inflammatory and immune response. It is known that phosphoryla-

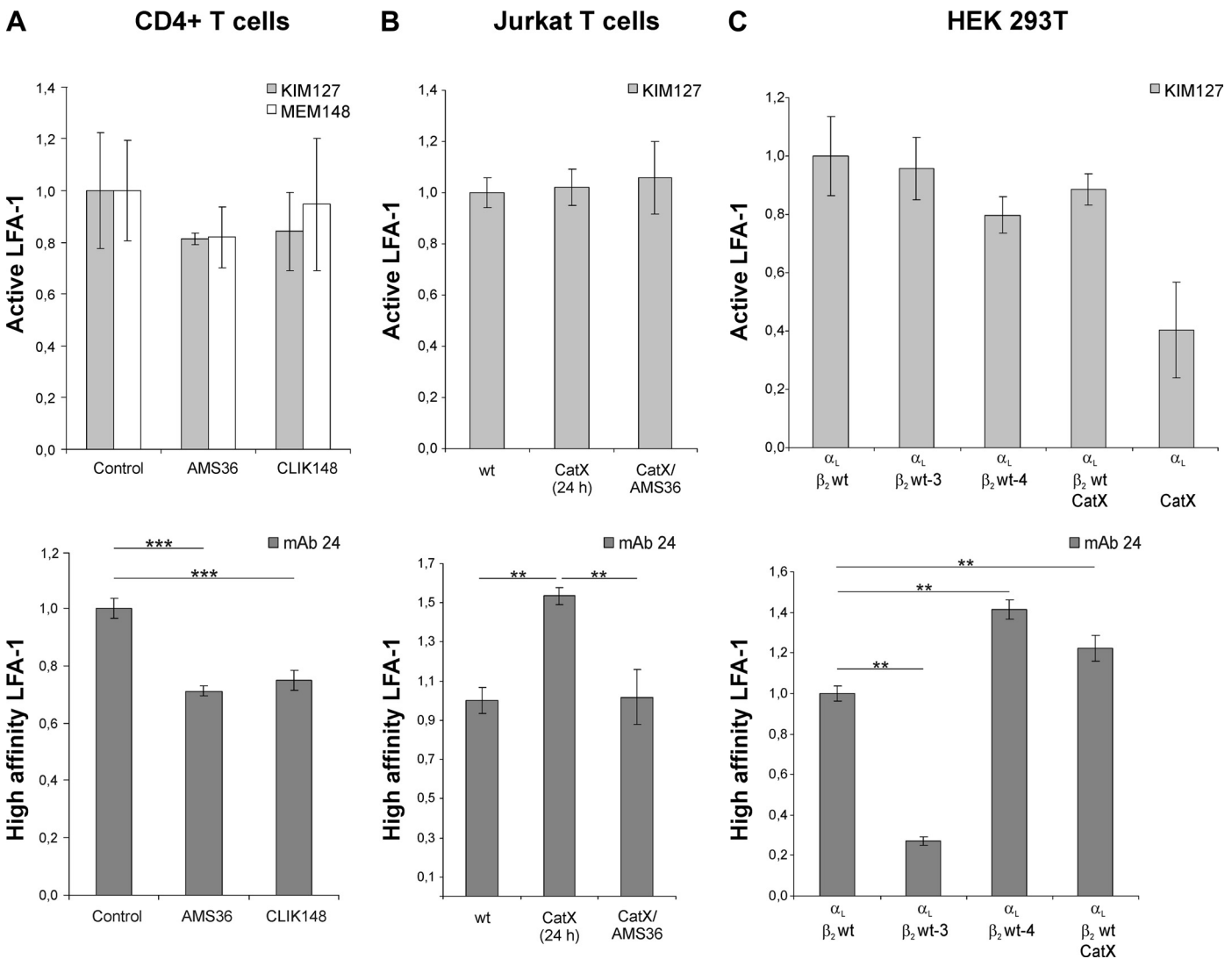

Figure 5. The influence of CatX on LFA-1 activation and affinity. Human $CD4^+$ T cells (A) and wt and CatX-overexpressing Jurkat cells (B) were pretreated with AMS36 and CLIK148 and labeled with conformation reporter mAb. The expression of different LFA-1 conformations was measured by flow cytometry. LFA-1 activation was detected by mAb KIM127 and MEM-148 (upper panels) and the expression of high-affinity LFA-1 by mAb 24 (lower panels). The values are related to those obtained for the control $CD4^+$ T cells without inhibitors or wt Jurkat cells (arbitrarily set to 1) and are representative of four independent experiments. (C) HEK293T cells were transiently transfected with α_L, β_2, and CatX expression constructs (the combinations of transfection constructs are indicated), and LFA-1 conformations were detected by flow cytometry using conformation reporter mAb. (Upper panel) The values represent the expression of active LFA-1 (KIM127-positive LFA-1). (Lower panel) The values represent the portion of active LFA-1 (KIM127-positive LFA-1), which is in the high-affinity conformation (mAb 24-positive LFA-1). All of the values are related to those obtained for wt LFA-1 (α_L/ β_2 wt; arbitrarily set to 1) and result from three independent experiments. Values are means ± SD (***P<0.001; **P<0.01).

tion of the β_2 integrin cytoplasmic tail plays a major role in the docking of various adaptor proteins to LFA-1. Phosphorylation of T758 acts as a molecular switch to inhibit filamin binding and allow 14-3-3 protein binding to the β_2 integrin cytoplasmic tail [29]. Post-translational modification of β_2 integrins by the carboxypeptidase CatX was proposed recently as another possibility for regulating the binding of adaptor proteins to the β_2 integrin cytoplasmic tail. CatX was shown in vitro to progressively cleave the first four C-terminal amino acids, S769, E768, A767, and F766, until P764 is present at the P2 position of the β_2 integrin cytoplasmic tail peptide [18, 23]. Truncation of the β_2 integrin cytoplasmic tail in Jurkat T cells overexpressing CatX was linked with enhanced migratory capacity and extreme morphological perturbations [13]. The present study confirms that CatX-dependent modification of the β_2 integrin cytoplasmic tail indeed occurs in T cells. By high-resolution SDS-PAGE, we identified a smaller β_2 integrin form, most likely corresponding to a 4-aa shorter β_2 subunit, whose level was dependent on CatX activity.

Talin, a cytoskeleton-associated protein, is directly involved in LFA-1 activation by regulating integrin affinity and clustering [30]. For β_3 integrins, it has been shown that the spatial

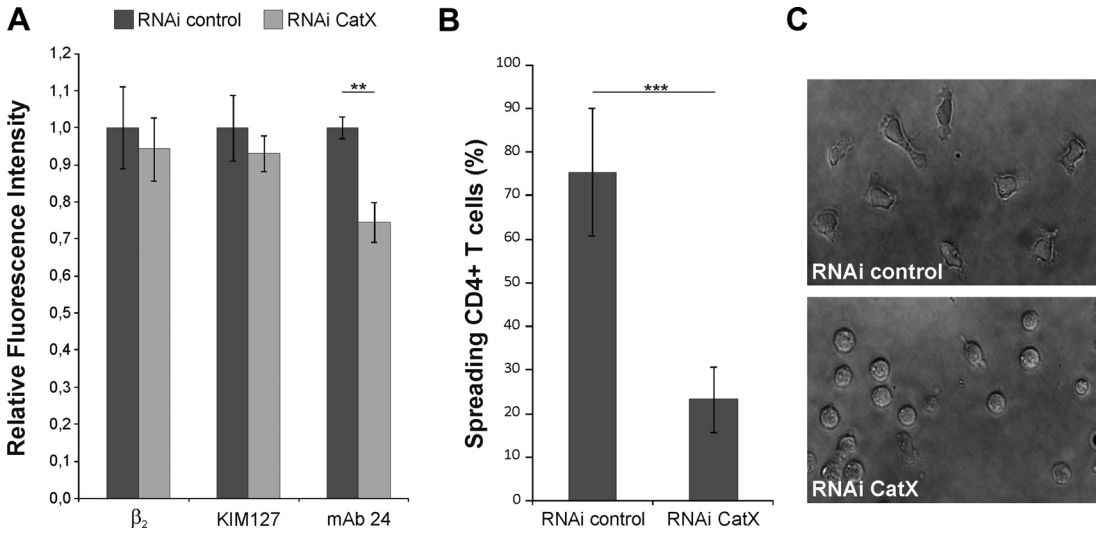

Figure 6. The effect of CatX silencing on LFA-1 affinity and cell spreading. The expression of different LFA-1 conformations was detected by conformation reporter mAb and flow cytometry. RNAi-mediated silencing of CatX in $CD4^+$ T cells had no effect on total LFA-1 (β_2) and active LFA-1 (KIM127), and the levels of high-affinity LFA-1 were reduced significantly (mAb 24). (B) $CD4^+$ T cells were plated on ICAM-1/SDF-1 and examined for the spreading phenotype after 24 h. At least 300 cells were analyzed/condition in two separated experiments. The ability of $CD4^+$ T cells to spread on the ICAM-1/SDF-1-coated surface was strongly limited after RNAi silencing of CatX. Values are means ± SD (***$P<0.001$; **$P<0.01$). (C) Representative images of control and CatX-silenced $CD4^+$ T cells spreading on ICAM-1/SDF-1.

separation of the α and β cytoplasmic tails, leading to unfolding of the bent conformation and β_3 integrin activation, is induced by the association of talin with the MP-binding site located at the β cytoplasmic tail [9]. This model of activation may be extrapolated to β_2 integrin LFA-1 activation, given that higher talin expression has been shown to induce the separation of LFA-1 cytoplasmic tails with concomitant activation of LFA-1 to bind ICAM-1 [16]. Gradual removal of the C-terminal amino acids from the β_2 tail by CatX intensifies the binding affinity for the talin head domain, as determined by SPR analysis. Further, coimunoprecipitation of talin with the β_2 integrin subunit was reduced after CatX-specific inhibition or silencing in Jurkat T cells. A molecular model of modified β_2 tail peptides in complex with talin F3 revealed that CatX cleavage induces conformational changes of the MD talin-binding site, affecting residues N749 and D750 vicinal to the NPLF motif and promoting the interaction intensity of the complex. The NPLF motif, which is conserved in the integrin β subunits, has been shown to be important for integrin affinity, cytoskeletal interactions, adhesion, and shape change [31, 32]. In T cells, all of these activities are affected by the activity of CatX [14, 25]. In addition, our findings demonstrate an essential requirement for CatX in regulating LFA-1-mediated T cell spreading on ICAM-1.

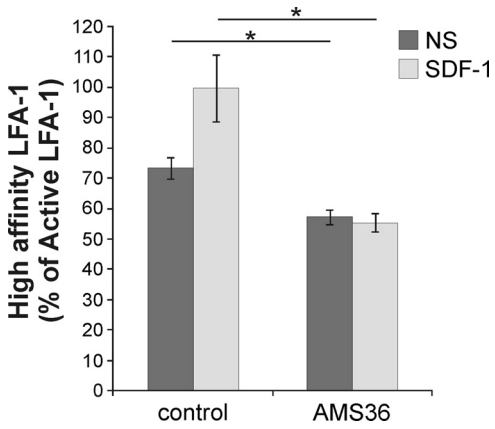

Figure 7. The influence of SDF-1 on the ability of CatX to modulate LFA-1 affinity. Jurkat cells were pretreated with AMS36 and stimulated or not (NS) by SDF-1. Cell surface levels of active LFA-1 (KIM127) and high-affinity LFA-1 (mAb 24) were measured by flow cytometry. The values represent the percentage of active LFA-1 ($KIM127^+$), which is in high-affinity conformation ($KIM127^+mAb24^+$). Two independent experiments were preformed. Values are means ± SD (*$P<0.05$).

LFA-1 can adopt three different conformations, a low (bent conformation)-, intermediate-, or high-affinity (extended conformation) state [17]. LFA-1 is regularly found in multiple conformations that can be mobile or stabilized by cytoskeletal adaptors. The complex equilibrium between these different conformations already exists in resting T cells and is shifted toward an immobilized high-affinity conformation on stimulation with PMA [28]. This conformational equilibrium could be regulated by proteolytic cleavage of the β_2 integrin cytoplasmic tail. By analyzing the activation and affinity epitopes of KIM127 and mAb 24 in the extracellular domain of the β_2 subunit in T cells, we showed that CatX does not affect the conversion of bent, inactive LFA-1 to the extended ligand-binding conformation. However, it appears that CatX cleavage alters the affinity of the portion of LFA-1 that is in the extended conformation. This is supported by the fact that modification of the β_2 tail by CatX does not alter the binding of

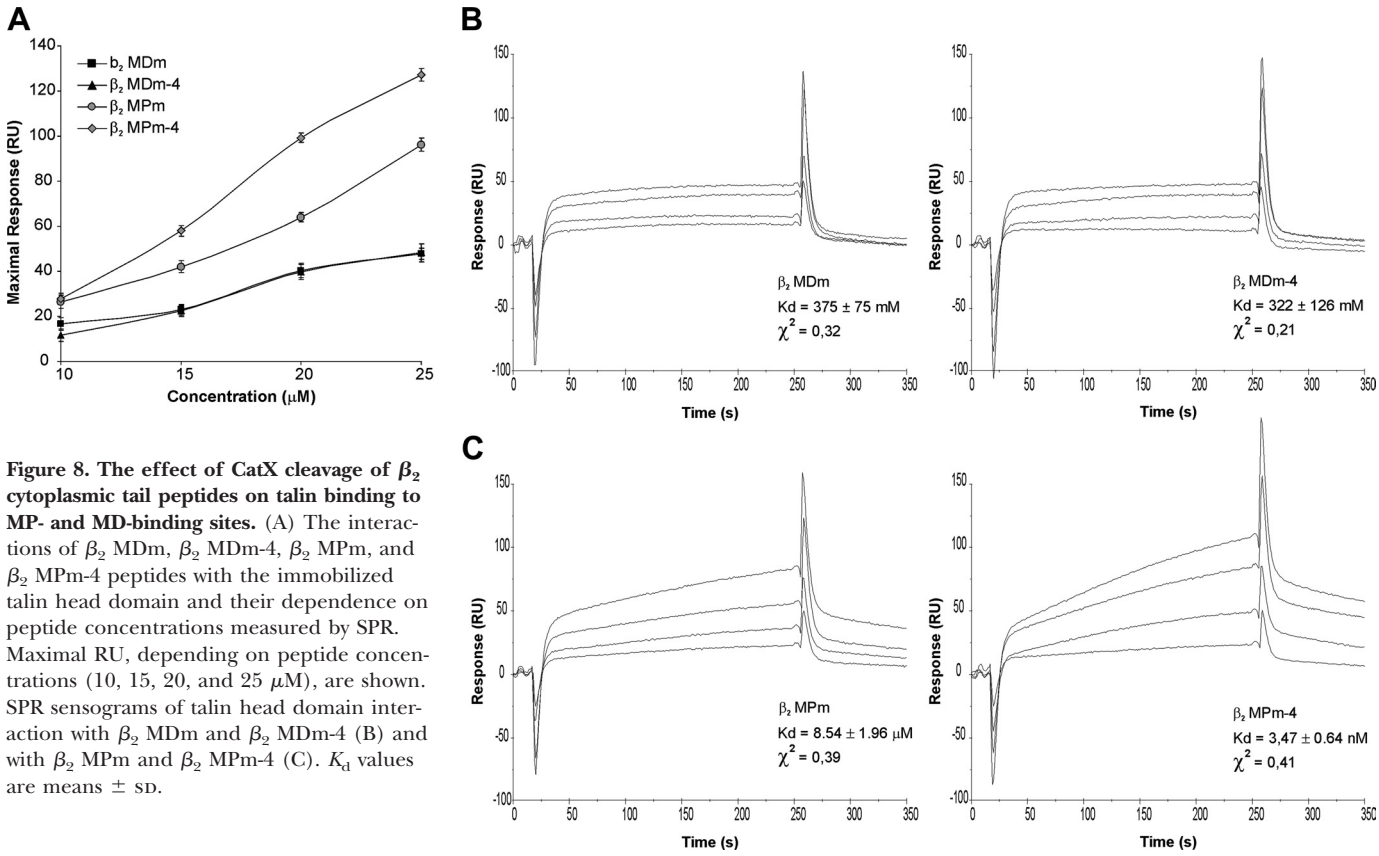

Figure 8. The effect of CatX cleavage of β_2 cytoplasmic tail peptides on talin binding to MP- and MD-binding sites. (A) The interactions of β_2 MDm, β_2 MDm-4, β_2 MPm, and β_2 MPm-4 peptides with the immobilized talin head domain and their dependence on peptide concentrations measured by SPR. Maximal RU, depending on peptide concentrations (10, 15, 20, and 25 μM), are shown. SPR sensograms of talin head domain interaction with β_2 MDm and β_2 MDm-4 (B) and with β_2 MPm and β_2 MPm-4 (C). K_d values are means ± SD.

talin F3 to the MP-binding site, which is responsible for integrin activation. On the contrary, CatX greatly affects the binding of talin F3 to the MD-binding site, suggesting that this interaction could indeed be responsible for the transition between different affinity conformations of extended LFA-1.

Reports describing talin association with different LFA-1 affinity conformations are controversial. In one study, talin was proposed to stabilize the intermediate-affinity LFA-1 [33], whereas in another, it was reported to associate with high-affinity LFA-1 in the focal zone of migrating T cells [34]. Our results suggest that talin binds to both extended conformations, although the affinity of β_2 integrin for talin is conformation-dependent. By cleaving the first three amino acids, S769, E768, and A767, from the C-terminal of the β_2 cytoplasmic tail, CatX increases the binding affinity for talin and promotes intermediate-affinity LFA-1. High-affinity LFA-1 is triggered by subsequent cleavage of the fourth amino acid, F766, providing an additional increase in affinity for talin. There is substantial evidence that intermediate-affinity LFA-1 is connected with another adaptor protein, α-actinin-1 [35], and we have shown that α-actinin-1 preferentially associates with β_2 wt-3 [β_2 (Δ767-769)] [14]. These two cytoskeletal proteins could be simultaneously bound to the β_2 subunit of intermediate-affinity LFA-1, given that the talin MD-binding site does not overlap with the α-actinin-1-binding site [36].

In summary, we have demonstrated that proteolytic cleavage of the β_2 cytoplasmic tail of integrin receptor LFA-1 by CatX regulates the binding of the adaptor protein talin and consequently, the function of its receptor. Sequential cleavage of C-terminal amino acids from the β_2 integrin cytoplasmic tail does not affect the activation of LFA-1; however, it promotes the transition from the intermediate- to a high-affinity state, which is necessary for tight binding of ligands, such as ICAM-1. Besides talin, CatX processing may affect the binding of other cytoskeletal adaptor proteins, such as α-actinin-1, together triggering the stepwise transition to high-affinity LFA-1 (**Fig. 9**). Our results reveal CatX as a possible target for the design of therapeutics for treatment of autoimmune and malignant diseases aimed at modulating LFA-1 affinity while avoiding loss of function.

AUTHORSHIP

Z.J. designed, preformed, analyzed all experiments unless stated otherwise and wrote the paper; N.O. contributed to design of research and discussed the data; B.D. prepared expression constructs; S.T. and S.G performed molecular modeling; U.Š. prepared primary cells; S.H and M.T contributed to design of research; and J.K. discussed the data and contributed to writing the paper.

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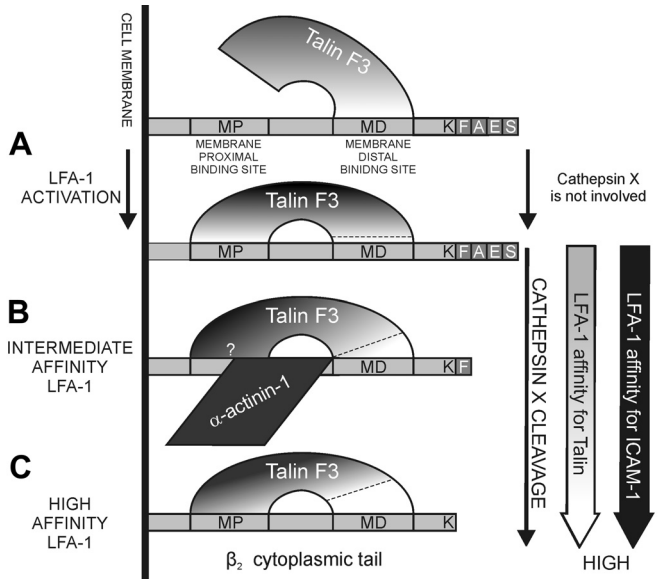

Figure 9. A simplified model of LFA-1 fine-tuning by CatX. (A) Talin F3/MD interaction provides an initial, strong linkage between talin and integrin, and the activation arises from the subsequent talin F3/MP interaction (proposed by Wegener et al. [9] for β_3 integrin activation). (B) CatX cleavage of the first three C-terminal amino acids from the activated LFA-1 progressively increases β_2 tail affinity for talin and α-actinin-1, promoting the intermediate-affinity LFA-1 conformation. (C) Subsequent cleavage of the fourth C-terminal amino acid (F766) further intensifies talin affinity for the MD-binding site, while reducing the affinity for α-actinin-1. This final β_2 tail processing step yields high-affinity LFA-1.

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carboxypeptidase · activation · cytoplasmic tail