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Lack of nuclear translocation of cytoplasmic domains of IL-2/IL-15 receptor subunits

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Abstract

Some sensors of extracellular signaling molecules such as Notch and sterol response element binding protein (SREBP) receive ligand-induced intra-membrane proteolysis followed by nuclear translocation of their cytoplasmic domains to regulate gene expression programs in the nucleus. It has not been extensively examined whether ligand-induced intra-membrane proteolysis of type I cytokine receptors and nuclear translocation of cytoplasmic domains occur. Here, by using a sensitive reporter system, we examined this possibility for the interleukin-2 (IL-2) receptor (IL-2R) β -chain (IL-2R β) and the IL-15 receptor (IL-15R) α -chain (IL-15R α). Flowcytometric analysis revealed that ligand stimulation does not induce nuclear translocation of their cytoplasmic domains. In addition, overexpression of the cytoplasmic domain of the common cytokine receptor γ -chain (γ c) in an IL-2R-reconstituted Ba/F3-derived cell line did not affect any biological responses including cell survival, disproving potential roles of the cleaved cytoplasmic domain of γ c as a signal transducer. Collectively, these results indicated that potential nuclear function of cleaved type I cytokine receptor subunits is not plausible.

Keywords

cytokine; regulated intra-membrane proteolysis; nuclear translocation; inducible translocation trap

1. Introduction

Some sensors of extracellular and intracellular signaling molecules receive ligand-induced intra-membrane proteolysis followed by nuclear translocation of their cytoplasmic domains to regulate gene expression programs in the nucleus [1]. For example, binding of Delta-like ligands to Notch induces a proteolytic cascade, ultimately resulting in release of Notch intracellular domain (NICD). NICD enters into the nucleus and acts as a component of transcriptional activator complex. Another example is SREBP, which is released by the proteolysis of a membrane protein precursor within the membrane in response to changes in internal sterol concentrations and regulates gene expression in the nucleus [2,3].

Accumulating evidence suggests critical importance of the Jak/Stat pathway in signal transduction from type I and type II cytokine receptors [4,5]. On the other hand, other signaling pathways are also activated upon cytokine binding [6], and it is possible that these receptors

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may also use different modes of signal transduction. In this regard, it has not been extensively examined whether ligand-induced intra-membrane proteolysis of type I cytokine receptors and nuclear translocation of cytoplasmic domains occur.

Here, by using a sensitive reporter system, we examined this possibility for IL-2R β and IL-15R α . Flowcytometric analysis revealed that ligand stimulation does not induce nuclear translocation of their cytoplasmic domains. In addition, overexpression of the cytoplasmic domain of γ c in an IL-2R-reconstituted Ba/F3-derived cell line did not affect any biological responses including cell survival, disproving potential roles of the cleaved cytoplasmic domain of γ c as a signal transducer. Collectively, these results indicated that potential nuclear function of cleaved type I cytokine receptor subunits is not plausible.

2. Materials and Methods

2.1. Plasmids construction

For construction of Notch1-LGV/pMXs expressing the fusion protein of human Notch1 and LGV, the *Hind* III - *Sfi* I fragment (7.3 kbp) and the *Sfi* I - *Bsp*E I fragment (371 bp) of the pcDNA3-1-hNotch1-FLAG plasmid [7] were cloned into the pMXs vector together with *Xmn* I- and *Sal* I-digested LGV and a double-strand oligonucleotide consisting of 5'-GATCCGGAGGCC TTCAA -3' and 5'-TTGAAGGCTCCG -3'.

LGV (NLS+)/pMX is a retroviral construct expressing the LGV (NLS+) protein consisting of DNA-binding domain (DB) and the dimerization domain of LexA, GFP and VP16 transactivation domain (TA). It was shown that the dimerization domain of LexA contains a cryptic nuclear localization signal (NLS) [8].

For construction of IL-2R β -LGV/pMX expressing the fusion protein of mouse IL-2R β and the LGV protein consisting of LexA DB, GFP, and VP16 TA, the carboxyl (C)-terminal region of mouse IL-2R β cDNA was amplified by PCR using mouse IL-2R β WT/pBS [9] as template and 5'-aggctgccccgaggatctcccccac-3' and 5'-aatcccgaggattagtgaggactgaatcttgggc-3' as primers. The PCR product was subcloned into the pCR2.1 vector (Invitrogen) and verified by sequencing. The cDNA encoding the LGV protein was digested from pLGV-N, which is a modified version of pLGV [10], with *Xmn* I and *Bam*H I and inserted into *Sma* I- and *Bam*H I-cleaved pCR2.1 plasmid containing the C-terminal region of mouse IL-2R β cDNA. The resultant plasmid was digested with *Bam*H I, the ends were filled-in with T4 DNA polymerase (DNA Blunting kit, Takara), and subsequently digested with *Apa* I. The cDNA encoding the fusion protein of the C-terminal region of mouse IL-2R β and LGV was ligated into the pMX vector [11] together with the cDNA encoding the amino-terminal region of mouse IL-2R β derived from mIL-2R β WT/pBS. LGV does not contain any NLS.

For construction of IL-15R α -LGV/pMX expressing the fusion protein of mouse IL-15R α and LGV, mouse IL-15R α cDNA [12] was amplified by PCR using 5'-actaaagcttggtcactgtggtggacaattggcc-3' and 5'-caaaccggggctcctgtgtcttcctccttgc-3' as primers. The PCR product was digested with *Eco*R I and subcloned into pBluescript SK (+) and verified by sequencing. The resultant plasmid was cleaved with *Bam*H I and *Sma* I, and the *Xmn* I- and *Bam*H I-digested LGV cDNA was inserted. The cDNA encoding a fusion protein of mouse IL-15R α and LGV was inserted into pMX.

For construction of IL-2R β /pMX expressing mouse IL-2R β , IL-2R β cDNA was inserted into pMX.

For construction of HA- γ c (cyto)/pEF expressing the HA-tagged cytoplasmic domain of human γ c (HA- γ c (cyto)), cDNA encoding a region of the cytoplasmic domain of γ c cleaved from

pIL-2R γ 2 [13] with *Nsp* I, which was blunted, and *Eco*R I was inserted into pEF-HA [14] digested with *Xba* I, which was filled in, and *Eco*R I, together with phosphorylated oligonucleotides generated by annealing 5'-ggccgcactggaacggacgatgccccg-3' and 5'-aattcggggcatcgtccgtccagtgc-3'.

For construction of HA- γ c (cyto)/pUHD10-3 expressing HA- γ c (cyto) in a tetracycline-inducible manner, cDNA encoding a region of the cytoplasmic domain of γ c cleaved from pIL-2R γ 2 with *Nsp* I, which was blunted, and *Eco*R I was inserted into pUHD10-3 [15] digested with *Xba* I, which was filled in, and *Eco*R I. The resultant plasmid was cleaved with *Eco*R I and inserted with the fragment encoding the HA-tag and a portion of the cytoplasmic domain of γ c derived from *Eco*R I-digested HA- γ c (cyto)/pEF.

2.2. Cell lines

BL2 is a Ba/F3-derived cell line containing the LexA-hCD2 reporter gene [10]. F7-rtTA3 is derived from F7, which is a Ba/F3-derived cell line expressing human IL-2R β , and expresses rtTA from the transfected pUHD172-1 plasmid [15].

2.3. Retroviral gene transduction

Retroviral gene transfer was performed as previously described [10].

2.4. Establishment of stable transformants

Establishment of stable transformants was done as previously described [10]. Briefly, 100 μ g of pUHD172-1 or HA- γ c (cyto)/pUHD10-3 was linearized with *Xho* I or *Pvu* I, respectively, and transfected into 1×10^7 of F7 or F7-rtTA3 cells together with the linearized puromycin- or hygromycin-resistant gene, respectively, by electroporation. Transfected cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3B-conditioned medium in the presence of puromycin (0.75 μ g/ml) or hygromycin B (1 mg/ml).

2.5. Flowcytometry

Flowcytometric analysis of GFP and hCD2 expression was performed as previously described [10].

2.6. Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as described previously [16]. Antibodies (Abs) used for immunoblot analysis in this study are anti-IL-2R β (sc-672), anti- γ c (sc-668), anti-Sp1 (sc-59), anti-Rho GDI (sc-360, Santa Cruz Biotechnology), anti-LexA (06-719), anti-RNA polymerase II (05-623, Upstate Biotechnology), anti-CD4 (Leu3a), and anti-HA (12CA5) Abs.

2.7. Northern blot analysis

Northern blot analysis of the cytoplasmic domain of human γ c gene was performed as described previously [14,17,18].

2.8. Cell death assay

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and human IL-2 (10 ng/ml) in the absence or presence of doxycycline (DOX) (10 μ g/ml) for 24 hr. Subsequently, cells were cultured in media that did not contain IL-2. Cell viability was examined by propidium iodide (PI) staining and flowcytometry.

3. Results and discussion

3.1. Detection of nuclear translocation of NICD by ITT

To address the issue of potential ligand-induced nuclear translocation of the cytoplasmic domains of type I cytokine receptors, we used a sensitive human CD2 (hCD2) reporter system of the inducible translocation trap (ITT) system [10]. This system consists of the LexA-hCD2 reporter gene composed of hCD2 with multiple LexA-binding sites and fusion proteins of cytokine receptor subunits and LGV, a fusion protein of LexA DB, GFP, and VP16 TA (Fig. 1A). If cytoplasmic domains of cytokine receptor subunits are cleaved upon ligand stimulation and translocate into the nucleus, LGV binds to the LexA-binding elements of the LexA-hCD2 reporter and induces expression of hCD2.

To examine whether this system can detect nuclear translocation of cytoplasmic domains of cell surface receptors, we generated a retroviral construct expressing the human Notch1 [7] fused with LGV (Notch1-LGV, Fig. 1A) and transduced into the BL2 cell line. BL2 is a Ba/F3-derived cell line containing the LexA-hCD2 reporter gene [10]. Expression of GFP was clearly detected by flowcytometry (Fig. 1B), showing the expression of the Notch1-LGV fusion protein. We treated transduced cells with EDTA, which depletes calcium and has been reported to activate Notch receptors [19]. In the absence of EDTA treatment, background expression of hCD2 was observed (Fig. 1C), suggesting spontaneous activation of Notch1 or activation by endogenous Notch ligand possibly expressed on Ba/F3-derived cell lines. EDTA treatment increased the percentages of hCD2^{high} populations in a dose-dependent manner (Fig. 1C and D), indicating increase in the amounts of NICD in response to calcium depletion. These results showed that the ITT system is sensitive enough to detect nuclear translocation of cytoplasmic domains of cell surface receptors induced by intra-membrane cleavage.

3.2. Lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-2R β

Next, we examined possible nuclear translocation of IL-2R β . IL-2R β is a shared subunit of IL-2R and IL-15R and a member of the type I cytokine receptor family [5]. LGV (NLS+)/pMX (positive control), a retroviral construct expressing LGV (NLS+), which constitutively resides in the nucleus and activates reporter expression, or IL-2R β -LGV/pMX, a retroviral construct expressing the fusion protein of mouse IL-2R β and the LGV protein (Fig. 2A), was transduced into BL2 cells. Transduced cells were cultured in the presence of IL-3 (1 ng/ml) or IL-2 (10 ng/ml) for two days, and GFP expression as well as hCD2 reporter expression was analyzed by flowcytometry. IL-2R β -LGV-transduced BL2 cells proliferated in response to IL-2 (data not shown), indicating that IL-2R β -LGV retains the ability to transmit proliferative signal. As shown in Fig. 2B, comparable expression levels of LGV (NLS+) and IL-2R β -LGV were observed. On GFP (+) BL2 cells expressing LGV (NLS+), hCD2 expression was readily detected (Fig. 2C and D). In contrast, no hCD2 reporter expression was detected on GFP (+) BL2 cells expressing IL-2R β -LGV cultured in the presence of IL-3 or IL-2 (Fig. 2C and D). This result showed that ligand-induced nuclear translocation of the cytoplasmic domain of IL-2R β does not occur.

To further confirm lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-2R subunits, we performed immunoblot analysis of cytoplasmic domains of IL-2R β and γ_c . Ba/F3 cells expressing mouse IL-2R β were growth factor-starved for 4 hrs and stimulated with mouse IL-2 (10 ng/ml) for the indicated time intervals. Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis with anti-IL-2R β , anti- γ_c , anti-Sp1, or anti-Rho GDI Ab. As shown in Fig. 3, cytoplasmic domains of γ_c were not detected following IL-2 stimulation. There was one main band (55 kDa) in the anti-IL-2R β blot of the nuclear extracts (Fig. 3). At this stage, it is not clear whether this band is a cleaved cytoplasmic domain of IL-2R β or non-specific bands. However, intensity of this band did not change by IL-2

stimulation (Fig. 3), arguing against its functional roles in signal transduction. These data collectively confirmed lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-2R subunits.

3.3. Lack of IL-15-induced nuclear translocation of the cytoplasmic domain of IL-15R α

Next, we examined possible nuclear translocation of IL-15R α . IL-15R α is the IL-15-specific subunit of IL-15R [20]. It does not belong to the type I cytokine receptor family but is structurally related to IL-2R α [20]. LGV (NLS+)/pMX (positive control) or IL-15R α -LGV/pMX, a retroviral construct expressing the fusion protein of mouse IL-15R α and the LGV protein (Fig. 4A), was transduced into BL2 cells ectopically expressing mouse IL-2R β by transduction of IL-2R β /pMX. Transduced cells were cultured in the presence of IL-3 (1 ng/ml) or IL-15 (10 ng/ml) for two days, and GFP expression as well as hCD2 reporter expression was analyzed by flowcytometry. IL-15R α -LGV-transduced BL2 cells expressing IL-2R β proliferated in response to IL-15 (data not shown), indicating that IL-15R α -LGV retains the ability to transmit proliferative signal. As shown in Fig. 4B, comparable expression levels of LGV (NLS+) and IL-15R α -LGV were observed. On GFP (+) cells expressing LGV (NLS+), hCD2 expression was readily detected. In contrast, marginal hCD2 reporter expression identical to that for GFP (–) population was detected on GFP (+) cells expressing IL-15R α -LGV cultured in the presence of IL-3 or IL-15 (Fig. 4C and D). This result showed that ligand-induced nuclear translocation of the cytoplasmic domain of IL-15R α does not occur.

To further confirm lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-15R subunits, we performed immunoblot analysis of cytoplasmic domains of IL-2R β and γ_c . Ba/F3 cells expressing mouse IL-2R β and mouse IL-15R α fused with LGV were growth factor-starved for 4 hrs and stimulated with human IL-15 (10 ng/ml) for the indicated time intervals. Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis with anti-LexA, anti-IL-2R β , anti- γ_c , anti-Sp1, or anti-Rho GDI Ab. As shown in Fig. 5, cytoplasmic domains of γ_c were not detected following IL-15 stimulation. There were one main band (55 kDa) and a faint band (43 kDa) in the anti-IL-2R β blot of the nuclear extracts (Fig. 5). At this stage, it is not clear whether these bands are cleaved cytoplasmic domains of IL-2R β or non-specific bands. However, intensities of these bands did not change by IL-15 stimulation, arguing against their functional roles in signal transduction. These data further confirmed the lack of IL-15-induced nuclear translocation of the cytoplasmic domain of IL-15R subunits.

3.4. Lack of biological activities of the overexpressed cytoplasmic domain of γ_c

In Notch signaling, NICD functions as an active form and overexpression of NICD induces malignant transformation [21]. As a complementary approach to the above-mentioned reporter-dependent detection of nuclear translocation of cytoplasmic domains of cytokine receptors, we examined whether cytoplasmic domains of cytokine receptors function as active forms using γ_c as an example. The cytoplasmic domain of γ_c was fused with the HA-tag (HA- γ_c (cyto)) (Fig. 6A). Co-transfection assay showed that HA- γ_c (cyto) retains the ability to bind Jak3 (Fig. 6B). We established IL-2R-reconstituted F7-derived clones that express HA- γ_c (cyto) in a tetracycline-dependent manner (Fig. 6C). To examine biological effects of overexpression of HA- γ_c (cyto), cells cultured in the presence of IL-2 were growth factor-starved in the absence or presence of doxycycline (Dox), and their viability was examined by PI staining and flowcytometry. As shown in Fig. 6D, there was no difference in cell viability after IL-2-starvation between Dox (–) and Dox (+) cells. Cell proliferation was also not affected by Dox-induced expression of HA- γ_c (cyto) (data not shown). Thus, overexpression of HA- γ_c (cyto) did not show any biological effects tested.

3.5. No evidence of nuclear translocation of cytoplasmic domains of type I cytokine receptor subunits

Here, we examined possible nuclear translocation of type I cytokine receptor subunits and its potential significance in signal transduction. ITT-based reporter assay was sensitive enough to detect nuclear translocation of NICD induced by intra-membrane cleavage. Our results clearly showed that there is no nuclear translocation of IL-2R β and IL-15R α in response to IL-2 and IL-15 stimulation, respectively. Another approach using overexpression of the cytoplasmic domain of γ c did not show any indication of its biological activities. Thus, it is not likely that type I cytokine receptors use a similar signal transduction mechanism to that utilized by Notch and SREBP.

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Figure 1.

Detection of nuclear translocation of NICD by ITT. **(A)** Scheme of human Notch1 fused with LGV (Notch1-LGV). **(B)** Expression of Notch1-LGV. Notch1-LGV/pMXs was transduced into BL2 as described previously [10]. Expression of GFP was compared between GFP (–) population (black line) and GFP (+) population (green). **(C)** Expression of hCD2 in transduced BL2 cells. Cells were treated with different concentrations of EDTA for 15 min at 37°C. 6 hr later, cells were non-stained (black line) or stained with phycoerythrin (PE)-conjugated anti-hCD2 Ab (red line), and hCD2 expression was analyzed on GFP (+) populations. **(D)** Dose-dependent increase in hCD2^{high} population by EDTA treatment of Notch1-LGV expressing BL2 cells.

Figure 2.

Lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-2Rβ. (A) IL-2R expression was reconstituted in BL2 cells by ectopic expression of IL-2Rβ-LGV. Potential cleavage and nuclear translocation of the cytoplasmic domain of IL-2Rβ can be detected by hCD2 reporter expression. (B) Expression of LGV (NLS+) and IL-2Rβ-LGV. LGV (NLS+)/pMX or IL-2Rβ-LGV/pMX was transduced into BL2 as described previously [10]. Expression of GFP was compared between GFP (-) population (black line) and GFP (+) population (green). (C) Expression of hCD2 in transduced BL2 cells. BL2 cells expressing IL-2Rβ-LGV were cultured in the presence of IL-3 (middle panel) or IL-2 (right panel) for two days. Cells were non-stained (black line) or stained with phycoerythrin (PE)-conjugated anti-hCD2 Ab (red

line), and hCD2 expression was analyzed on GFP (+) populations. **(D)** Expression levels of hCD2 were normalized against those of GFP.

Figure 3.

Lack of IL-2-induced nuclear translocation of the cytoplasmic domain of IL-2Rβ. Ba/F3 cells expressing mouse IL-2Rβ were growth factor-starved for 4 hrs and stimulated with mouse IL-2 (10 ng/ml) for the indicated time intervals. Cytoplasmic (C) and nuclear extracts (N) (5 μg) were subjected to immunoblot analysis with anti-IL-2Rβ, anti-γc, anti-Sp1, or anti-Rho GDI Ab.

Figure 4.

Lack of IL-15-induced nuclear translocation of the cytoplasmic domain of IL-15R α . (**A**) IL-15R expression was reconstituted in BL2 cells expressing mouse IL-2R β by transduction of IL-15R α -LGV. Potential cleavage and nuclear translocation of the cytoplasmic domain of IL-15R α can be detected by hCD2 reporter expression. (**B**) Expression of LGV (NLS+) and IL-15R α -LGV. LGV (NLS+)/pMX or IL-15R α -LGV/pMX was transduced into BL2 expressing IL-2R β as described previously [10]. Expression of GFP was compared between GFP (-) population (black line) and GFP (+) population (green). (**C**) Expression of hCD2 in transduced BL2 cells expressing IL-2R β . Cells expressing IL-15R α -LGV were cultured in the presence of IL-3 (middle panel) or IL-15 (right panel) for two days. Cells were non-stained

(black line) or stained with PE-conjugated anti-hCD2 Ab (red line), and hCD2 expression was analyzed on GFP (+) populations. **(D)** Expression levels of hCD2 were normalized against those of GFP.

Figure 5. Lack of IL-15-induced nuclear translocation of the cytoplasmic domain of IL-15Rα. Ba/F3 cells expressing mouse IL-2Rβ and mouse IL-15Rα fused with LGV were growth factor-starved for 4 hrs and stimulated with human IL-15 (10 ng/ml) for the indicated time intervals. Cytoplasmic (C) and nuclear extracts (N) (5 μg) were subjected to immunoblot analysis with anti-LexA, anti-IL-2Rβ, anti-γc, anti-RNA polymerase II (Pol II), or anti-Rho GDI Ab.

Figure 6.

Overexpression of the cytoplasmic domain of γc in an IL-2R-reconstituted Ba/F3-derived cell line did not affect cell viability after IL-2 withdrawal. **(A)** Scheme of HA-tagged cytoplasmic domain of human γc (HA- γc (cyto)). **(B)** Physical interaction of HA- γc (cyto) and Jak3. COS cells were co-transfected with Jak3 cDNA as well as an expression construct of CD4 γ /pEF, pEF-HA or HA- γc (cyto)/pEF. Whole cell lysates were subjected to immunoprecipitation with anti-CD4 or anti-HA Ab. Immunoprecipitates were analyzed by immunoblot analysis with anti-Jak3 Ab. **(C)** Induction of HA- γc (cyto) by DOX. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and mouse IL-3 (1 ng/ml) in the absence or presence of DOX (5 μ g/ml) for 24 hr. Total RNA was purified and subjected to Northern blot analysis using the probe for the cytoplasmic domain of human γc . Purification of total RNA and Northern blot analysis was performed as previously described [14]. **(D)** Cell death by withdrawal of IL-2. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and human IL-2 (10 ng/ml) in the absence or presence of DOX (10 μ g/ml) for 24 hr. Subsequently, cells were cultured in media that did not contain IL-2. Cell viability was examined by PI staining and flowcytometry.