Ubiquitin-like polypeptide conjugates to acceptor proteins in concanavalin A- and interferon γ -stimulated T-cells

Morihiko NAKAMURA¹ and Yoshinori TANIGAWA

Department of Biochemistry, Shimane Medical University, 89-1 Enya-cho, Izumo 693, Japan

Monoclonal non-specific suppressor factor (MNSF), a lymphokine produced by a murine T-cell hybridoma, possesses pleiotrophic non-specific suppressive functions. MNSF β (a subunit of MNSF) is a 14.5 kDa fusion protein consisting of a protein with 36% homology with ubiquitin and ribosomal protein S30. The ubiquitin-like segment of MNSF β (Ubi-L) is an 8 kDa polypeptide with MNSF-like activity. Since the amino acids critical for the ubiquitination process are conserved in Ubi-L, we examined whether Ubi-L may conjugate with intracellular proteins in a manner similar to the ubiquitin system. Rabbit polyclonal antibodies specific for Ubi-L detected the induction of Ubi-L conjugations, including 33.5 kDa and 70 kDa molecules in concanavalin A (Con A)-stimulated T-cells, but not in

lipopolysaccharide-stimulated B-cells and macrophages. High-molecular-mass conjugates were consistently present in pan-T-cells. However, free Ubi-L could not be observed in all the cells tested. Con A-activated CD8⁺ T-cells, but not CD4⁺ T-cells, induced the 70 kDa Ubi-L adduct, which was recognized by an anti-MNSF monoclonal antibody. Treatment of CD8⁺ T-cells with interferon (IFN) γ also caused the expression of the 70 kDa Ubi-L adduct, whereas the responses to IFN α and IFN β were nil. Antigen- and Con A- stimulated D.10 G4.1, a murine T helper clone type 2, induced the 33.5 kDa, but not the 70 kDa, adduct. These results suggest a role for Ubi-L conjugation in the regulation of T-cell activation.

INTRODUCTION

Ubiquitin, a highly conserved 76-amino-acid protein present in all eukaryotic cells, is involved in the removal of abnormal, misfolded or improperly assembled proteins [1]. The process is accomplished through a unique post-translational modification in which the C-terminus (Gly-Gly doublet) of ubiquitin is covalently ligated to lysine residues in target proteins [2]. Ubiquitin-dependent proteolysis is carried out via a multi-enzyme, ATP-dependent degradative pathway [3]. Other cellular processes in which the ubiquitin system is also involved include DNA repair [4], cell-cycle progression [5], ribosome biogenesis [6], stress response [7], apoptosis [8] and regulation of the activation of the transcription factor nuclear factor κ B [9]. In addition, several signal-transducing receptors, in particular the ζ subunit of the TCR-CD3 (T-cell receptor-cluster of differentiation 3) complex [10] and the high-affinity IgE receptor [11], are ubiquitin target proteins. Interestingly, ubiquitin itself has been shown to exhibit an inhibitory effect on IgE-induced platelet cytotoxicity [12,13]. Interferon (IFN)-stimulated human lymphocytes and monocytes have been found to release a 15 kDa ubiquitin-cross-reactive protein (UCRP), which has significant homology with ubiquitin [14]. Although its function remains largely uncertain, UCRP is suspected to be involved in a cell-to-cell signalling role. These observations imply that ubiquitin and ubiquitin-like proteins may be part of a previously unrecognized modulatory mechanism of the immune responses.

We have previously isolated and characterized the monoclonal non-specific suppressor factor (MNSF), a 70 kDa product of a murine T-cell hybridoma (E17), which inhibits the generation of

lipopolysaccharide (LPS)-induced Ig-secreting cells [15] and division in various tumour cell lines of murine origin [16,17]. We have cloned a cDNA encoding a subunit of MNSF, which was termed MNSF β [18]. MNSF β cDNA encodes a protein of 133 amino acids consisting of a ubiquitin-like protein (36% identity with ubiquitin) fused to the ribosomal protein S30 [18]. The ubiquitin-like moiety (Ubi-L) may be cleaved from MNSF β in the cytosol, as has been demonstrated for the rat Fau protein in reticulocyte lysates [19]. We have also shown that the recombinant form of Ubi-L expressed in bacteria has biological activity similar to that of the E17-derived MNSF [20]. Since C-terminal amino acids of ubiquitin (Gly-Gly), involved in the isopeptide bond formation during conjugation, are conserved in Ubi-L, we were motivated to determine whether Ubi-L may conjugate to intracellular proteins in a way similar to the ubiquitination process. In the present study, murine lymphoid cells and cell lines were employed, since the nature of MNSF has been extensively characterized in these cells. Our results revealed that concanavalin A (Con A) can remarkably induce Ubi-L conjugations in T-cells and T-cell lines. We also describe the possible involvement of IFN γ in the Ubi-L conjugation.

MATERIALS AND METHODS

Animals

Female Balb/c mice, 8–16 weeks of age, were obtained from Clea Japan (Osaka, Japan). All of the mice were reared in the Laboratory of the Institute for Experimental Animals, Shimane Medical University, Izumo, Japan.

Abbreviations used: IFN, interferon; UCRP, ubiquitin-cross-reactive protein; NSF, non-specific suppressor factor; MNSF, monoclonal NSF; hNSF, human NSF; LPS, lipopolysaccharide; Ubi-L, ubiquitin-like polypeptide; Con A, concanavalin A; Ab, antibody; mAb, monoclonal Ab; ECL, enhanced chemiluminescence; GST, glutathione S-transferase.

¹ To whom correspondence should be addressed.

Materials

Ubiquitin and rabbit anti-ubiquitin antibody (Ab) were obtained from Sigma (St. Louis, MO, U.S.A.). Mouse recombinant IFN γ (1×10^7 units/mg) was purchased from Genzyme (Cambridge, MA, U.S.A.). Mouse IFN α (1×10^7 units/ml) was obtained from SeroTech (Tokyo, Japan). A specific Ab against a synthetic peptide (PU1) corresponding to the ubiquitin-like region was raised in rabbits as previously described [18].

MNSF and Ubi-L

Hybridoma (E17)-derived 70 kDa MNSF was prepared by affinity chromatography with the use of an anti-MNSF monoclonal Ab (mAb) (MO6), as previously described [21]. Recombinant Ubi-L was obtained as previously described [20]. Briefly, Ubi-L was expressed as a fusion protein with glutathione S-transferase (GST) using the pGEX-2T vector (Pharmacia, Piscataway, NJ, U.S.A.). Ubi-L was cleaved from the fusion partner by thrombin and purified by using an anti-PU1 Ab coupled to Sepharose 4B (Pharmacia).

Preparation of T-cells, B-cells and macrophages

T-cells

T-cells were enriched from splenocytes by Lymphoprep centrifugation, passage over nylon wool, and treatment with a mixture of mAbs against B220 and MAC-1, and complement. All Ab depletions resulted in a population of $> 99\%$ T-cells, as assayed by flow cytometry. To separate CD4 $^+$ or CD8 $^+$ single positive subsets, the cells attached to the nylon-fibre column were treated with a cocktail of mAb plus either anti-CD4 (GK1.5) or anti-CD8 (116-3.1), and complement. The Ab depletions resulted in $< 1\%$ of the respective cell population remaining.

B-cells

Splenocytes were incubated with a mixture of mAbs against Thy-1.2, L3T4, Lyt-2, MAC-1 and a granulocyte-specific antigen, and complement (Thy-1.2, L3T4 and Lyt-2 are T-cell-specific antigens; MAC-1 is a macrophage-specific antigen). Live cells were separated from dead cells and erythrocytes on Histopaque. With this procedure, the T-cell-depleted population was consistently $> 95\%$ B-cells (B220 $^+$).

Macrophages

For the preparation of peritoneal exudate macrophages, male Balb/c mice, aged 8–12 weeks, were injected intra-peritoneally with 2 ml of thioglycollate broth (Difco, Detroit, MI, U.S.A.), and peritoneal exudate cells were harvested after 4 days.

Tumour cell lines

Various tumour cell clones of murine origin, including EL4 (T lymphoma), BW5147 (T lymphoma), D.10 (type-2 helper T-cell clone), MOPC-31C (plasmacytoma), WEHI-231 (B lymphoma), J774 (macrophage-like cell clone) and CFTL-15 (mast cell clone) cells were maintained in our laboratory, and we examined the Ubi-L conjugation in these cells.

Cell culture

Cultures were grown at 37 °C in a humidified 5% CO $_2$ atmosphere. Cells were cultured at a density of 5×10^5 – 2×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of the following reagents: Con A (Calbiochem, La Jolla, CA, U.S.A.), LPS (Difco), IFN γ and IFN α .

Immunoblot analysis

Cells were washed with 25 mM potassium phosphate-buffered saline (pH 7.3), followed by lysis in $1 \times$ SDS sample buffer [22]. The resulting cell extracts were sonicated for 15 s, heated at 90 °C for 5 min, and then resolved by SDS/PAGE, under reducing conditions in polyacrylamide gels. Gels were equilibrated for 10 min in transfer buffer [20 mM Tris base, 144 mM glycine and 20% (v/v) methanol] before electroblotting. The gels were blotted electrophoretically (6 h at 12 V/cm) on Immobilon-P membrane (Millipore, Tokyo, Japan), which was subsequently blocked with 10 mM Tris/HCl/150 mM NaCl containing 5% (w/v) BSA and 1% (w/v) ovalbumin. The membranes were incubated with anti-PU1 IgG (5 g/ml) in the blocking buffer, after which they were incubated with peroxidase-conjugated anti-rabbit IgG (Capel, Durham, NC, U.S.A.). Excess reagents were washed out with 10 mM Tris/HCl/150 mM NaCl/0.05% (w/v) Nonidet-P. Detection was done according to the enhanced chemiluminescence (ECL) detection system (Amersham).

RESULTS

A specific Ab against Ubi-L does not cross-react with ubiquitin

Prediction of the secondary structure of Ubi-L by the Chou-Fasman rules [23] confirms the tandem largely hydrophobic structural units. The recombinant form of Ubi-L exhibits a high tendency to aggregate and precipitate during purification, probably due to the hydrophobic region, residues 29–70. This probably accounts for our consistently poor yield of Ubi-L after cleavage from its fusion partner GST. In contrast, ubiquitin has a stable secondary structure containing only a low percentage of α -helix or β -sheet [24]. To detect the potential conjugation of Ubi-L in mouse splenocytes, a specific antibody against a synthetic internal 12-amino-acid peptide (PU1) of Ubi-L with low homology with ubiquitin was raised in rabbits [18]. To demonstrate that the anti-PU1 Ab specifically recognizes Ubi-L, immunoblot analysis was performed. The anti-PU1 Ab detected recombinant Ubi-L, whereas it failed to recognize ubiquitin (Figure 1, lanes 1 and 2). Conversely, anti-ubiquitin Ab bound to ubiquitin, but not to Ubi-L (Figure 1, lanes 3 and 4). These results clearly demonstrate that proteins detected with anti-PU1 Ab are Ubi-L and/or its conjugated forms and preclude the possibility of the cross-reaction of this antibody with ubiquitin.

Figure 1 Immunoreactivity of anti-PU1 Ab with Ubi-L on immunoblots

Equal amounts (300 ng) of Ubi-L and ubiquitin were separated on SDS/15% polyacrylamide slab gels. The proteins were blotted on Immobilon-P membranes and were allowed to react with anti-PU1 Ab or anti-ubiquitin Ab and then with the peroxidase-conjugated IgG of goat anti-rabbit IgG. Antigenic bands were revealed by the ECL system. Lanes 1 and 2, anti-PU1 Ab; lanes 3 and 4, anti-ubiquitin Ab; lanes 1 and 3, Ubi-L; lanes 2 and 4, ubiquitin. The positions of molecular-mass markers (kDa) are shown on the left-hand side.

Figure 2 Detection of conjugated Ubi-L in Con A-activated splenocytes

(A) Triplicate cultures of splenocytes were incubated for 48 h in the absence or presence of $3 \mu\text{g/ml}$ Con A and then washed and lysed in SDS sample buffer, as described in the Materials and methods section. Pooled samples representing 3×10^5 cells were resolved by SDS/PAGE (12% polyacrylamide) under reducing conditions and then transferred to Immobilon-P. Blots were allowed to react with $5 \mu\text{g/ml}$ affinity-purified anti-Ubi-L (anti-PU1) and then with the peroxidase-conjugated IgG of goat anti-rabbit IgG. Lane 1, unstimulated splenocytes; lane 2, Con A-stimulated splenocytes. Detection was done by the ECL system. The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively. (B) To estimate the size of the HM, SDS/PAGE (8% polyacrylamide) was performed under reducing conditions.

Con A induces the Ubi-L conjugation in splenocytes

To determine the presence of significant Ubi-L conjugates in female Balb/c mice splenocytes, cell lysates were resolved by SDS/PAGE and subjected to electrophoretic transfer and immunochemical staining for Ubi-L conjugates. Several very weak bands were detected by anti-PU1 Ab (Figure 2A, lane 1),

Figure 3 The specificity of Ubi-L conjugation in Con A-activated T-cells

Splenocytes were separated into T-cells, B-cells and macrophages as described in the Materials and methods section. T-cells were stimulated with $3 \mu\text{g/ml}$ Con A. B-cells and macrophages were stimulated with 20 and $1 \mu\text{g/ml}$ LPS respectively. These cells were cultured for 48 h, and the induction of Ubi-L was assessed as described in the legend to Figure 2. Samples representing 2×10^5 cells were resolved by SDS/PAGE (12% polyacrylamide) under reducing conditions. Lanes 1 and 2, separated T-cells; lanes 3 and 4, B-cells; lanes 5 and 6, macrophages. Lanes 1, 3 and 5, unstimulated cells; lanes 2, 4 and 6, mitogen-stimulated cells. The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively.

Figure 4 Time course for Ubi-L conjugation in Con A-activated T-cells

Top: triplicate confluent cultures of T-cells were incubated in the presence of Con A ($3 \mu\text{g/ml}$) for various periods of time and then lysed in SDS sample buffer. Lane 1, unstimulated T-cells; lane 2, cultured for 6 h; lane 3, 12 h; lane 4, 24 h; lane 5, 48 h; lane 6, 72 h. Pooled triplicate samples representing 1×10^5 cells were resolved by SDS/PAGE and the Ubi-L adducts were revealed, as described in the legend to Figure 2. The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively. Bottom: the average of the three experiments after densitometric analysis of the bands is shown; ●, 70 kDa MNSF; ○, 33.5 kDa Ubi-L adduct. Data are expressed as means $\pm$ S.D.

suggesting that Ubi-L might conjugate to acceptor proteins in unstimulated cells. Free Ubi-L was not detected, implying that Ubi-L may promptly conjugate to acceptors following the cleavage from MNSF β in the cytosol. The anti-PU1 Ab detected increased levels of 33.5 kDa and 70 kDa conjugates in extracts from splenocytes following 48 h incubation with $3 \mu\text{g/ml}$ of Con A relative to those in extracts from unstimulated cells (Figure 2A, lane 2). The delayed accumulation of these conjugates did not result from a general increase in total protein, because a parallel gel stained with Coomassie Brilliant Blue showed no significant change in the pattern of protein bands (results not shown). It should be noted that high-molecular-mass conjugates (≈ 180 kDa) were ubiquitously observed (Figures 2A and 2B, HM) in T-cells.

Ubi-L conjugation is specific for T-cells

We next investigated whether the Ubi-L conjugation is specific for activated T-cells. Splenocytes were separated into T-cells, B-cells and macrophages as described in the Materials and methods section. As shown in Figure 3, the pattern of Ubi-L conjugation in Con A-activated T-cells was qualitatively identical with that seen in Con A-activated splenocytes. The high-molecular-mass conjugates were observed in both unstimulated and activated T-cells. B-cells and macrophages were cultured for 48 h in the presence of LPS and tested for Ubi-L conjugation. There was no

Figure 5 Ubi-L conjugation in CD4⁺ and CD8⁺ T-cells

CD4⁺ and CD8⁺ T-cells were prepared as described in the Materials and methods section. Triplicate confluent cultures of the T-cell subsets were incubated for 48 h in the presence of Con A (3 μ g/ml). Pooled triplicate samples representing 5×10^4 cells were resolved by SDS/PAGE (12% polyacrylamide) under reducing conditions, and the Ubi-L conjugation was assessed immunochemically as described in the legend to Figure 2. Lanes 1, 2 and 3, CD4⁺ T-cells; lanes 4, 5 and 6, CD8⁺ T-cells. Lanes 1 and 4, unstimulated cells; lanes 2 and 5; cultured for 24 h; lanes 3 and 6, 48 h; lane 7, immunostaining of lane 5 with the MO6 mAb. The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively.

increase in the corresponding Ubi-L adducts in these cells, thereby implying that Ubi-L conjugation might be specific for activated T-cells. B-cells and macrophages were stimulated with various concentrations of LPS (0.1–50 μ g/ml) for different times (12–72 h), resulting in a pattern similar to that shown in Figure 3 (results not shown). Therefore, we conclude that Ubi-L preferentially conjugates to some acceptors in Con A-activated T-cells. Several minor immunoreactive bands were consistently present in all the cells tested, in good agreement with the fact that the expression of Ubi-L mRNA is ubiquitous [18]. A significant difference of the pattern of Ubi-L conjugations in Con A-activated T-cells due to sex or age was not observed (results not shown).

In preliminary experiments identifying the optimal number of T-cells to induce prominent Ubi-L conjugations, we noted that 5×10^5 T-cells/ml of culture resulted in a maximal production. In addition, a concentration of 3 μ g/ml Con A yielded a large amount of the 33.5 kDa and the 70 kDa conjugates (results not shown), so it was used for the following experiments. We next performed kinetics studies to elucidate the underlying mechanism of Ubi-L conjugation in T-cells. T-cells were stimulated with Con A for various periods (6–72 h), resulting in a maximal 70 kDa conjugate at 24 h incubation. In contrast, a notable band at 33.5 kDa was contained in the T-cells cultured for 48 h. The 33.5 kDa band was observed even after the 72 h incubation, whereas the 70 kDa band was not seen (Figure 4), in good accordance with the previous observations showing the extra-cellular release of 70 kDa MNSF [15].

Induction of the 70 kDa Ubi-L adduct is limited to CD8⁺ T-cells

We have reported that the release of human NSF (hNSF), a counterpart of mouse MNSF, may be limited to CD8⁺ T-cells [25]. To determine whether Ubi-L adduct formation is limited to the CD8⁺ T-cell subset, T-cells were separated into CD4⁺ and CD8⁺ T-cells and cultured in the presence of Con A. An increase in the amount of the 33.5 kDa conjugate was observable in both T-cell subsets, whereas the 70 kDa conjugate corresponding in mobility to MNSF was restricted to CD8⁺ T-cells (Figure 5, lane 5). Lane 7 shows the immunostaining of lane 5 by using an anti-

Figure 6 Ubi-L conjugation in various tumour cell lines of murine origin

Cell lines grown in suspension culture were lysed in SDS sample buffer, and Ubi-L conjugation was assayed as described in the legend to Figure 2. D.10 cells (2×10^4 cells/well) were stimulated with conalbumin (100 μ g/ml) or Con A (2 μ g/ml) plus interleukin-1 β (0.5 unit/ml) for 48 h. Each lane represents 1×10^5 cells. Cell lines: lane 1, EL-4; lane 2, BW5147; lane 3, D.10; lane 4, conalbumin-stimulated D.10; lane 5, Con A- and interleukin-1 β -activated D.10; lane 6, MOPC-31C; lane 7, WEHI-231; lane 8, J774; lane 9, CFTL-15. The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively.

MNSF mAb (MO6). MO6 recognized the band at 70 kDa in Con A-activated CD8⁺ T-cells. As previously noted [18], the authentic 70 kDa MNSF consists of α subunit, which is detected by MO6, and β subunit (Ubi-L). Collectively, the 70 kDa protein is MNSF, and the machinery of this Ubi-L adduct formation may be limited to CD8⁺ T-cells. The high-molecular-mass conjugation-protein bands were observed in all T-cells. We speculate that the high-molecular-mass conjugates could be involved in unrecognized mechanism(s) other than immune suppression.

T-cell lines are able to induce Ubi-L conjugations

An explorative attempt was made to detect Ubi-L conjugation in various cells of murine origin. Among a series of mouse cell lines tested, high-molecular-mass conjugates were seen in thymoma cell lines, EL4 and BW5147; however, neither cell line showed any detectable levels of bands at 33.5 kDa and 70 kDa (Figure 6, lanes 1 and 2). A type-2 helper T-cell line (D.10) also showed the high-molecular-mass conjugates without any stimulation. This cell line was further tested, since the response to Ubi-L had been well characterized in our previous study [17]. Con A-activated D.10 cells contained a prominent band at 33.5 kDa, but not at 70 kDa (Figure 6, lane 4). D.10 cells showed the corresponding response to antigen (conalbumin) (Figure 6, lane 5), although signalling pathways triggered by two distinct stimulations may differ [26]. Accordingly, the high-molecular-mass conjugates may be contained in pan-T-cells and the 33.5 kDa conjugate might be limited to activated T-cells. It should be pointed out that these T-cell lines lack the ability to produce the 70 kDa MNSF. By the use of these cell lines, isolation and characterization of the high-molecular-mass conjugates are under way in our laboratory to identify the acceptor proteins. The Ubi-L adducts were not seen in non-T-cell lines, including MOPC-31C, WEHI231, J774 and CFTL-15 (Figure 6, lanes 6–9).

IFN γ induces the 70 kDa Ubi-L adduct in CD8⁺ T-cells as well as Con A

We previously demonstrated that IFN γ -activated T-cells produce 70 kDa MNSF [21]. To interpret the intracellular accumulation of the MNSF, the potential ligation of Ubi-L in IFN γ -activated

Figure 7 Ubi-L conjugation in response to different IFN types

The conjugation of Ubi-L in response to various concentrations of IFN γ and different interferon types was assessed as described in the legend to Figure 2. Triplicate confluent cultures of T-cells (2×10^5 cells) were incubated for 48 h in the presence of IFN γ (lane 1, unstimulated T-cells; lane 2, 5 units/ml of IFN γ -activated cells; lane 3, 20 units/ml; lane 4, 50 units/ml; lane 5, 100 units/ml) or in the presence of type I IFNs (lane 7, 20 IU/ml of IFN α -activated cells; lane 8, 100 IU/ml; lane 9, 20 IU/ml of IFN β ; lane 10, 100 IU/ml). T-cells were also stimulated with 3 μ g/ml Con A in the presence of 100 μ g/ml anti-IFN γ mAb (lane 6). The mobilities of the high-molecular-mass conjugates (HM), 70 kDa MNSF and 33.5 kDa Ubi-L adduct and the molecular-mass standards (kDa) are indicated to the right and left of the Figure respectively.

T-cells was examined. The 70 kDa Ubi-L conjugation (MNSF) was induced in the IFN γ -activated T-cells, in a dose-dependent manner, with 20 units/ml IFN γ being the most effective (Figure 7, lane 4). It is known that Con A-stimulated T-cells produce IFN γ . Thus it is conceivable that the Con A-induced IFN γ may secondarily elicit Ubi-L conjugation. To test this possibility, an anti-IFN γ neutralizing mAb was added to the cultures together with Con A. This mAb remarkably inhibited the induction of the 70 kDa MNSF, but not of the 33.5 kDa protein (Figure 7, lane 6), indicating that IFN γ may be involved in the Con A-induced Ubi-L conjugation. IFN γ did not induce the 33.5 kDa conjugate at any concentrations (5–100 units/ml), leading to the suggestion that Con A may induce acceptor protein(s) not elicited by IFN γ . IFN type specificity for UCRP adducts in human lung carcinoma has been reported [27]. The structurally related type I IFNs, IFN α and IFN β , strongly elicit conjugated UCRP, whereas the structurally distinct type II IFN γ does not. We also examined the ability of IFN α and IFN β to induce Ubi-L conjugation in T-cells. There was no increase in the corresponding Ubi-L conjugates in extracts from T-cells incubated with a saturating amount of either IFN α or IFN β (Figure 7, lanes 7–10), during various periods of culture (results not shown).

DISCUSSION

The results presented in this paper are consistent with our hypothesis that Ubi-L may conjugate to intracellular proteins as well as ubiquitin. We have previously demonstrated that T-cell hybridoma-derived 70 kDa MNSF consists of Ubi-L and other component(s) [18] that may be involved in the T-cell receptor α chain [28]. In this context, it is interesting that Ubi-L preferentially conjugates to acceptor(s) in Con A-activated T-cells. Both MNSF and UCRP are extracellularly released from activated lymphoid cells and exhibit species-specific action [20,29]. These polypeptides are subjected to induction by IFN. Interestingly, they show type specificity for IFN and have immunoregulatory properties, although their functions are opposite. UCRP induces the proliferation of human B-depleted peripheral blood lymphocytes [29]. In contrast, hNSF, a human counterpart of MNSF, inhibits the proliferation of mitogen-stimulated human

peripheral blood mononuclear cells [28]. Since UCRP can elicit IFN γ in peripheral-blood-lymphocyte culture supernatants, hNSF might be secondarily induced. Thus it is possible that UCRP and MNSF/hNSF may be closely related to each other in the immune responses. It seems unlikely that IFN α and IFN β are involved in the induction of Ubi-L conjugations (Figure 7). We have demonstrated that IFN α exhibits a synergic action with IFN γ in terms of the inhibition of IgE production [30]. However, IFN α did not show any synergism with IFN γ in the Ubi-L conjugation (results not shown).

Several minor bands other than those migrating at the positions of 33.5 kDa and 70 kDa were constantly detected in the cells. However, the pattern of the Ubi-L conjugation is distinct from that seen with ubiquitin [31]. The distribution of ubiquitin conjugates is more heterogeneous, presumably due to a variety of acceptor proteins and the results of degradation of these proteins. As mRNA for Ubi-L is ubiquitously expressed, one may speculate that Ubi-L has substantially important functions in cytoplasm or nuclei as does ubiquitin.

UCRP conserves the ubiquitin LRLRGG C-terminus involved in isopeptide formation. The Arg-72 of ubiquitin is critical for ubiquitin-activating enzyme [32]. Ubiquitin is successively activated to a thiol ester of ubiquitin-activating enzyme, then transferred to a thiol group of a ubiquitin-carrier protein, and finally transferred to an internal Lys residue of a target protein. For some target proteins, this latter step requires a third enzyme, ubiquitin-protein ligase. Like UCRP, 125 I-labelled GST-Ubi-L conjugates to intracellular proteins *in vitro* [32a]. The Ubi-L GRMLGG C-terminus lacks the Arg residue, indicating that the mode of action of Ubi-L may differ from that of ubiquitin. However, other amino acids critical for ubiquitination [33] are conserved in Ubi-L [20]. Thus it is conceivable that some enzymes involved in the Ubi-L conjugation may exist and the underlying mechanism of action might be in parallel to the ubiquitination process.

The function of the 33.5 kDa Ubi-L adduct is unknown at present. This adduct is irrelevant to 70 kDa MNSF, because the MO6 mAb recognizing MNSF α subunit failed to react with it (Figure 5) and because an increase in the level of this adduct was not accompanied by an increase in the 70 kDa MNSF (Figure 4). It is possible that the association of Ubi-L to an unidentified 25 kDa acceptor protein might be implicated in the proliferation and/or differentiation of T-cells. Identification and characterization of this molecule are under way to clarify Ubi-L's action involved in T-cell process. It is interesting to note that high-molecular-mass conjugates are consistently observed in pan-T-cells. The amounts are unchanging during Con A or antigen activation. It is unclear whether the expression of the acceptor proteins for Ubi-L is specific for T-cells. T-cell tumour lines described above may be useful for the purification of these molecules. It is well known that polyubiquitination causes high-molecular-mass conjugates [34]. The polyubiquitination is involved in degradation of intracellular proteins including cyclin [5] and I κ B [35]. The possibility that Ubi-L conjugates to itself in a manner similar to the polyubiquitination process cannot be ruled out at present.

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