**Biochemical Analysis of the Receptor for Ubiquitin-like Polypeptide**

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Monoclonal nonspecific suppressor factor (MNSF), a lymphokine produced by murine T cell hybridoma, possesses pleiotropic antigen-nonspecific suppressive functions. A cDNA clone encoding MNSF-β, an isoform of the MNSF, has been isolated and characterized. MNSF-β cDNA encodes a fusion protein consisting of a ubiquitin-like segment (Ubi-L) and ribosomal protein S30. Ubi-L appears to be cleaved from the ribosomal protein and released extracellularly in association with T cell receptor-like polypeptide. In the current study we have characterized the biochemical nature of the Ubi-L receptor on D.10 G4.1, a murine T helper clone type 2. Biotinylated Ubi-L bound preferentially to concanavalin A-stimulated but not to unstimulated D.10 cells. Detergent-extracted membrane proteins were applied to an immobilized Ubi-L column. SDS-polyacrylamide gel electrophoresis of eluted fraction revealed a band of Mr = 82,000. Biotinylated Ubi-L specifically recognized this band, confirming that the 82-kDa protein is the Ubi-L receptor. A complex of Mr = 90,000 was visualized by immunoprecipitation of 125I-Ubi-L cross-linked to the purified receptor followed by SDS-polyacrylamide gel electrophoresis and autoradiography. In addition, a 105-kDa protein was communoprecipitated by anti-Ubi-L receptor (82-kDa polypeptide) antibody, indicative of the association of this protein with the Ubi-L receptor complex. Amino acid sequence analysis of the 82-kDa polypeptide revealed that the Ubi-L receptor may be a member of a cytokine receptor family.

Ubiquitin, a highly conserved 76-amino acid protein present in all eukaryotic cells, is involved in the degradation of short lived or structurally abnormal proteins. The process is accomplished through a unique posttranslational modification in which the carboxyl Gly-Gly terminus of ubiquitin is ligated covalently to lysine residues in acceptor proteins. Ubiquitin-dependent proteolysis is conducted via a multienzyme, ATP-dependent degradative pathway (1). Other cellular processes in which the ubiquitin system is also involved include antigen processing (2), ribosome biogenesis (3), cell cycle progression (4), and regulation of the transcriptional nuclear factor-κB (5). In addition, several signal transducing receptors, in particular the ζ-subunit of the TCR1-CD3 complex (6), the high affinity IgE receptor (7), and platelet-derived growth factor receptor (8, 9), are ubiquitinated receptors.

The monoclonal nonspecific suppressor factor (MNSF) is a product of a concanavalin A (ConA)-activated murine hybridoma that inhibits the generation of lipopolysaccharide (LPS)-induced immunoglobulin-secretory cells, proliferation of mitogen-activated T and B cells, and interleukin (IL)-4 secretion by bone marrow-derived mast cells (10, 11). We have cloned a cDNA encoding a subunit of MNSF (12). The subunit, termed MNSF-β, encodes a protein of 133 amino acids consisting of a ubiquitin-like protein (36% identity with ubiquitin) fused to the ribosomal protein S30. We have reported evidence showing that the ubiquitin-like segment of MNSF-β (Ubi-L) is responsible for its activity (13). Ubi-L inhibits IgE and IgG1 production by LPS-activated B cells and division in various tumor cell lines of murine origin (14).

Most recently, we have demonstrated that Ubi-L covalently conjugates to intracellular acceptor proteins in vitro (15) and in vivo (16). MNSF-α, a subunit of MNSF, was identified as an acceptor protein for Ubi-L. It is probable that Ubi-L might be released in a posttranslationally modified form (i.e. conjugation of Ubi-L to MNSF-α) because it lacks a signal peptide (12). Partially purified isopeptidase dissociates Ubi-L from MNSF-α, suggesting that the COOH-terminal Gly-Gly doublet of Ubi-L may covalently ligate to the lysine residue in MNSF-α (17).

Several other ubiquitin-like proteins have been isolated and characterized. Sentrin (also called SUMO-1), for instance, preferentially modifies nuclear proteins (18). Like ubiquitin, the ubiquitin cross-reactive protein (UCRP) conjugates to a number of intracellular proteins (19). Interestingly, UCRP and Ubi-L are subjected to induction by interferon (IFN) (11, 20). Furthermore, they show type specificity for IFN and have immunoregulatory properties (14, 21), although they have opposite functions (22). Accordingly, ubiquitin-like proteins may be involved in many biological reactions such as immune responses.

We have presented evidence that mitogen-activated T and B cells, and murine lymphoid cell lines, may have an MNSF receptor (23). Although further biochemical and functional analysis of this receptor protein had been prevented because of the lack of a recombinant ligand, Ubi-L enabled us to isolate and characterize the receptor for MNSF. In the present study, we describe how Ubi-L specifically binds to cell surface receptors on mitogen-activated lymphocytes and the T helper type 2 clone, the D.10 cell. Studies were also performed to characterize the biochemical nature of Ubi-L receptor protein.

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‡ The abbreviations used are: TCR, T cell receptor; MNSF, monoclonal nonspecific suppressor factor; ConA, concanavalin A; LPS, lipopolysaccharide; IL, interleukin; Ubi-L, ubiquitin-like segment of MNSF; UCRP, ubiquitin cross-reactive protein; IFN, interferon; Ab, antibody; GST, glutathione S-transferase; bio-Ubi-L, biotinylated Ubi-L; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; WGA, wheat germ agglutinin.
Receptor for Ubiquitin-like Polypeptide

EXPERIMENTAL PROCEDURES

Materials—Ubiquitin and rabbit anti-ubiquitin antibody (Ab) were obtained from Sigma (St. Louis, MO). Mouse recombinant IFN-γ (1 x 10^5 units/mg) was purchased from Genzyme (Cambridge, MA). Mouse IFN-α (1 x 10^5 units/ml) was obtained from ServoTech (Tokyo, Japan). Specific Ab against synthetic peptides corresponding to the ubiquitin-like region (PU) was raised in rabbits as described previously (12). Peroxidase-conjugated goat anti-mouse IgG Ab was from Capel (Durham, NC).

Tumor Cell Lines—D.10 (type 2 helper T cell clone), BW5147 (T line), EL4 (T lymphoma), MOPC-31C (plasmacytoma), NFS-SC-1 (pre-B lymphoma), L929 (fibroblast), and B16 (melanoma) were main-
ly lymphoma), EL4 (T lymphoma), MOPC-31C (plasmacytoma), NFS-5C-1 (Durham, NC).

Peroxidase-conjugated goat anti-mouse IgG Ab was from Capel (Durham, NC).

Binding Experiments of Biotinylated Ubi-L to Target Cells—Binding experiments were performed at 24 °C. Biotinylated Ubi-L (bio-Ubi-L; 20 nM) and the cells (2 x 10^5) to be tested were incubated in 200 μl of the binding medium (RPMI 1640 containing 1% bovine serum albumin and 20 mM Hepes, pH 7.4) for 2 h. Nonspecific binding, unless stated otherwise, was determined by measuring binding in the presence of 100-fold molar excess unlabeled Ubi-L. Specific binding was determined by subtracting nonspecific binding. The resulting binding was washed with binding medium five times and incubated with 200 μM of streptavidin-horseradish peroxidase conjugate in the same medium for an additional hour. The cells were then washed five times with 0.1 M sodium phosphate, pH 7.4, to a final concentration of 20 mM. Mouse anti-receptor serum or anti-Ubi-L receptor Ab. The beads were then packed into a column and incubated with sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 0.1% Triton X-100, and then the receptor was eluted with 1 x NaCl. The eluates were concentrated in Centricron-10 (Amicon, Beverly, MA) and subjected to reverse phase HPLC (Cosmosil, Nacalai tesq, Kyoto, Japan).

To obtain small peptides suitable for amino acid sequence analysis, purified Ubi-L receptor was digested with trypsin at an enzyme-substrate molar ratio of 1:100 at 37 °C for 4 h. Tryptic peptides were separated by reverse phase HPLC using a C18 column equilibrated in 0.1% trifluoroacetic acid and 50% acetonitrile in 0.1% trifluoroacetic acid. The peptides were then developed with a 5–50% linear gradient of acetonitrile for 120 min at 25 °C with a flow rate of 1.0 ml/min. Peptides were sequenced directly from polyvinylidene difluoride membranes using an ABI477 protein sequencer (Applied Biosystems).}

Immunoblotting—The biotinylated cell surface proteins (250 μg of protein) were applied to the Ubi-L column and incubated as described above. The receptor was eluted with a 2 x SDS sample buffer, subjected to 10% SDS-PAGE, and blotted onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in PBS for 1 h and then washed three times with PBS containing 0.5% Tween 20 (PBS/Tween 20). Subsequently, the membranes were incubated with streptavidin conjugated to horseradish peroxidase (1:1,000) in PBS/Tween 20 for 45 min. Membranes were washed five times in PBS/Tween 20 and developed using the ECL reagents (Amersham Pharmacia BioTech). In some experiments, MNF-β or ubiquitin was coupled to CNBr-activated Sepharose 4B and used for affinity chromatography as described above.

Ligand Blot Assay—Detergent-extracted membrane proteins were prepared from antigen-stimulated D.10 cells that were not labeled with biotin. The proteins (750 μg) were blotted onto nitrocellulose membranes as described above. The nitrocellulose sheets were incubated in buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20, and 50 μg/ml biotin-labeled Ubi-L or 200 μg/ml biotin-labeled ubiquitin for 1 h at 4 °C. The sheets were washed twice with binding buffer without the Ubi-L for 5 min and then incubated with streptavidin conjugated to horseradish peroxidase in PBS/Tween 20 for 30 min and subsequently washed for three times for 5 min with the same buffer. Detection of labeled proteins was performed with ECL reagents.

Immunization of Mice—A protein band (82 kDa) was excised from polyacrylamide slab gels, minced, and injected subcutaneously together with complete Freund's adjuvant. Additional immunizations were given 3 and 4 weeks later.

Immunoprecipitation of a Cross-linked 125I-Ubi-L Receptor Complex with Anti-Receptor Serum—Ubi-L was labeled with 125I as described previously for GST-Ubi-L (15). Aliquots of the purified receptor (500 ng) were mixed with 125I-Ubi-L and left for 1 h at 24 °C. Disuccinimidyl suberate was added to a final concentration of 0.3 mM. The cross-linking was stopped after 30 min at 4 °C by the addition of Tris-HCl buffer, pH 7.5, to a final concentration of 20 mM. Mouse anti-receptor serum or control serum was added and incubated for 2 h at 24 °C. The antigen-antibody complex was adsorbed on protein A-Sepharose and analyzed by SDS-PAGE followed by autoradiography. In some experiments, ConA-activated D.10 cells were biotinylated and solubilized as described under "Labeling of Cell Surface Receptors by Biotinylation." To determine whether proteins other than the 82-kDa polypeptide are associated with the Ubi-L receptor, lysates were immunoprecipitated with anti-Ubi-L receptor Ab.

Affinity Chromatography on a Wheat Germ Agglutinin (WGA) Column—Ubi-L receptor purified by a Ubi-L column was incubated at 4 °C for 6 h with 5 ml of WGA-Sepharose equilibrated in 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10% glycerol, and 0.1% Triton X-100. After incubation, the mixture was transferred to a column, the beads allowed to settle, and the column was washed with 50 ml of 0.5 mM KCl, 50 mM

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Tris-HCl, pH 7.4, 140 mM NaCl, and 0.1% Triton X-100. The absorbed Ubi-L receptor was then eluted with 10 ml of 0.5 mM N-acetyl-D-glucosamine, 50 mM Tris-HCl, pH 7.4, 0.5% NaCl, 10% glycerol, and 0.1% 

Deglycosylation of Ubi-L Receptor Proteins—The membrane fraction of ConA-activated D.10 cells was acidified by the addition of sodium citrate buffer, pH 6.2, to a final concentration of 50 mM. Neuraminidase (0.7 unit) was added, and the mixture was incubated at 37 °C for 4 h. SDS sample buffer was added to deglycosylated samples and boiled for 5 min. SDS-PAGE and immunostaining with anti-Ubi-L receptor Ab were carried out as described above.

Neutralizing Tests—Determination of IgE production by LPS-stimulated B cells was done as described previously (14). Briefly, the purified B cells (5 × 10^6/ml) were cultured with 20 µg/ml LPS. Ubi-L, recombinant IFN-γ, and anti-Ubi-L receptor serum (IgG) were added at the initiation of the cultures. Supernatants were harvested 7 days after initiation of the cultures, and IgE production was detected by enzyme-linked immunosorbent assay.

RESULTS

Binding Experiments of Recombinant Ubi-L to Target Cells—Previous experiments have shown that Ubi-L acts on murine helper T cell clone, D.10 cells (25). To investigate whether or not Ubi-L would bind specifically to D.10 cells, a binding assay was performed. Purified recombinant Ubi-L enabled acquisition of bio-Ubi-L with a high specific activity comparable to that of unlabeled Ubi-L. As shown in Fig. 1, bio-Ubi-L bound to ConA-activated D.10 cells (48 h). Each biotinylated polypeptide was added in the absence or presence of a 100-fold molar excess of unlabeled ligand. Specific binding was presented in the figure. The data shown are the means ± S.D. of three independent experiments.

Fig. 1. Binding of bio-Ubi-L to D.10 cells. The specific binding of 20 nM bio-Ubi-L (○, ■), 20 nM MNSF-β (□, △), and 100 nM ubiquitin (▲) at 24 °C to D.10 cells (2 × 10^6) was determined. ○, ■, □, △, unstimulated D.10 cells; ○, ■, □, △, ConA-activated (48 h). Each biotinylated polypeptide was added in the absence or presence of a 100-fold molar excess of unlabeled ligand. Specific binding was presented in the figure. The data shown are the means ± S.D. of three independent experiments.

Fig. 2. Binding of bio-Ubi-L to B cells and T cells. The specific binding of bio-Ubi-L (20 nM) at 24 °C to 3 µg/ml ConA-stimulated (48 h) T cells (5 × 10^6) (○) and 20 µg/ml LPS-stimulated B cells (5 × 10^6) (□) was determined. Specific binding is presented in the figure as described in the legend to Fig. 1. The data shown are the means ± S.D. of three independent experiments.

We next investigated the cellular distribution of the Ubi-L receptor. Binding experiments were carried out using various cells of murine and human origin. Among a series of mouse cell lines tested, EL4 and MOPC-31C cells apparently carried the Ubi-L receptors (Table I). Of note, both cell lines are sensitive to Ubi-L in terms of inhibition of proliferation (13). D.10 cells were stimulated with antigen (conalbumin) and ConA, as described previously (25). Ubi-L bound to both stimulated D.10 cells. Together, the expression of Ubi-L receptor should be limited to lymphoid cells. In contrast, Ubi-L did not bind to any human cell lines such as Jurkat, K562, MOLT-3, Namalwa, HL60, U937, Detroit 562 (data not shown), suggestive of the species specificity of Ubi-L action.

Specificity of Ubi-L Binding to Its Receptor—To clarify the specificity of the Ubi-L binding, competitive assay experiments were performed by adding to the experimental system the nonlabeled Ubi-L, MNSF-β, ubiquitin, and other suppressive cytokines such as IFN-γ and IL-10. As can be seen in Fig. 3, Ubi-L and MNSF-β exclusively inhibited the binding of bio-Ubi-L to ConA-activated D.10 cells. However, the irrelevant ligands (IFN-γ and IL-10) did not show any competition, indicative of the specificity for Ubi-L binding. Ubiquitin showed a slight but significant inhibition of the Ubi-L binding in agreement with previous finding that ubiquitin inhibits Ubi-L-induced suppression (13).

Affinity Chromatography and Immunoblotting—We next investigated biochemical nature of the receptor protein for Ubi-L. Cross-linking experiments with the use of recombinant Ubi-L were insufficient for identification of Ubi-L receptor protein(s). We observed heterogeneous bands on SDS-PAGE (data not shown), which seemed to be self-aggregation of Ubi-L probably

the homology. We also examined whether Ubi-L would recognize mitogen-activated lymphocytes. T cells and B cells were separated from splenocytes as described under “Experimental Procedures.” As shown in Fig. 2, exposure of the T cells to 3 µg/ml ConA for 2 days led to maximal binding of bio-Ubi-L on the cell surface. Bio-Ubi-L bound to 20 µg/ml LPS-stimulated B cells almost in the same manner as ConA-activated T cells. In contrast, neither unstimulated (day 0) T cells nor B cells showed any significant bio-Ubi-L binding. These results are consistent with those of binding experiments of native MNSF (23).

Ubi-L Receptor Expression Is Limited to Lymphoid Cells—We next investigated the cellular distribution of the Ubi-L receptor. Binding experiments were carried out using various cells of murine and human origin. Among a series of mouse cell lines tested, EL4 and MOPC-31C cells apparently carried the Ubi-L receptors (Table I). Of note, both cell lines are sensitive to Ubi-L in terms of inhibition of proliferation (13). D.10 cells were stimulated with antigen (conalbumin) and ConA, as described previously (25). Ubi-L bound to both stimulated D.10 cells. Together, the expression of Ubi-L receptor should be limited to lymphoid cells. In contrast, Ubi-L did not bind to any human cell lines such as Jurkat, K562, MOLT-3, Namalwa, HL60, U937, Detroit 562 (data not shown), suggestive of the species specificity of Ubi-L action.

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because of its strong hydrophobicity. We also employed $^{125}$I-GST-Ubi-L, which is a stable fusion protein. It should be noted that the activity of GST-Ubi-L is lower than that of Ubi-L. A trace amount of protein (approximately 120 kDa) was reproducibly cross-linked by $^{125}$I-GST-Ubi-L (data not shown). Therefore, we decided to use affinity chromatography on an immobilized Ubi-L column as the main step of Ubi-L receptor purification. D.10 cells were stimulated with ConA, biotinylated, and lysed. Biotinylated membrane proteins (250 mg/0.1 ml) were incubated with Ubi-L-Sepharose, and bound proteins were eluted as described under "Experimental Procedures." The eluates were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and visualized by ECL system. As depicted in Fig. 4A, only a single band of 82 kDa under nonreducing conditions was observed (lane 2). The migrated position of this band was unchanged under reducing conditions (lane 3). These results are consistent with the cross-linking experiments with the use of $^{125}$I-GST-Ubi-L (34 kDa) in terms of the molecular mass. On the contrary, no band was recovered from the biotinylated membrane proteins from unstimulated D.10 cells (lane 1), in good accordance with the results of binding assay with the use of $^{125}$I-GST-Ubi-L (34 kDa) in terms of the molecular mass.

### Table I

**Cellular distribution of murine Ubi-L receptor**

| Designation | Characteristics | Ubi-L bound$^a$ pmol/10^6 cells |
|-------------|----------------|-------------------------------|
| EL4         | T lymphoma     | 0.6 ± 0.3                     |
| MOPC-31C    | Plasmacytoma   | 0.5 ± 0.1                     |
| D.10        | Type 2 helper T cells clone | — | | |

Unstimulated

| antigen$^c$ | 0.7 ± 0.2 |
| Con$^d$     | 1.1 ± 0.1 |
| BW5147      | T lymphoma |
| NFS-5C-1    | Pre-B lymphoma |
| L929        | Fibroblast |
| B16         | Melanoma |

$^a$ Specifically bound Ubi-L was determined as described in the legend to Fig. 1.

$^b$ Less than 0.05 pmol/10^6 cells.

$^c$ D.10 cells were stimulated with conalbumin (100 μg/ml) for 48 h.

$^d$ D.10 cells were costimulated with 2 μg/ml ConA and 0.5 unit/ml IL-1β for 48 h.

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**FIG. 3.** Specificity of bio-Ubi-L binding to ConA-activated D.10 cells. D.10 cells (2 × 10^6 cells) were incubated with 20 nM bio-Ubi-L in the presence of unlabeled Ubi-L (●), MNSF-β (●), ubiquitin (■), IFN-γ (□), and IL-10 (▲). The binding of bio-Ubi-L to D.10 cells was determined as described under "Experimental Procedures." The data shown are the means ± S.D. of the three independent experiments.

**FIG. 4.** Biochemical analysis of Ubi-L receptor by SDS-PAGE. Panel A, SDS-PAGE of the purified Ubi-L receptor. D.10 cells (5 × 10^6 cells) were stimulated with ConA for 48 h, biotinylated, and lysed as described under "Experimental Procedures." Biotinylated membrane proteins were applied to a Ubi-L-Sepharose column, and bound proteins were eluted. The eluates were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and visualized by ECL system. Lane 1, unstimulated D.10 cells; lanes 2 and 3, ConA-stimulated (48 h); lanes 1 and 2, eluate from the Ubi-L-Sepharose column under nonreducing conditions; lane 3, same as lane 2 but under reducing conditions. The positions of molecular mass markers (kDa) are shown on the left. Panel B, ligand blot assay for Ubi-L receptor protein. Detergent-extracted membrane proteins were prepared from D.10 cells as described under "Experimental Procedures." The proteins were blotted onto nitrocellulose sheets and incubated with bio-Ubi-L (lanes 1 and 3) or biotinylated ubiquitin (lane 2). Lanes 1 and 2, extract from ConA-stimulated D.10 cells; lane 3, unstimulated cells. The positions of molecular mass markers (kDa) are shown on the left. Panel C, affinity chromatography on WGA. Ubi-L receptor purified by a Ubi-L-Sepharose column was applied to a WGA column. Fractions from WGA were electrophoresed on 10% gel and silver stained. Lane 1, effluent; lane 2, eluate.

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2 M. Nakamura and Y. Tanigawa, unpublished data.
(Fig. 1) and the previous observations that antigen-activated, but not unstimulated, D.10 cells are sensitive to Ubi-L (25). Additionally, we made MNSF-β and ubiquitin affinity columns to isolate Ubi-L receptor protein(s). The same band of 82 kDa was obtained from MNSF-β column, whereas no significant amount of protein band could be recovered from the ubiquitin column (data not shown). To confirm that the 82-kDa protein is a Ubi-L receptor, a ligand blot assay was carried out. Detergent-extracted membrane proteins were prepared from ConA-stimulated D.10 cells and blotted onto nitrocellulose membranes. Bio-Ubi-L specifically recognized the 82-kDa band (Fig. 4B, lane 1), suggesting that it should be a ligand for this receptor protein. In contrast, Ubi-L did not bind to any membrane proteins from unstimulated D.10 cells (Fig. 4B, lane 3). Interestingly, ubiquitin slightly bound to the Ubi-L receptor (Fig. 4B, lane 2). We also examined whether the 82-kDa Ubi-L receptor binds to lectins. Ubi-L receptor purified by a Ubi-L column was incubated with WGA-Sepharose. After 6 h, the absorbed Ubi-L receptor was eluted with N-acetyl-D-glucosamine. The eluate was electrophoresed and silver stained. As can be seen in Fig. 4C, the 82-kDa Ubi-L receptor was recovered in the eluate fraction, suggesting that Ubi-L receptor may bind to WGA.

We next performed immunoblotting analysis by the anti-receptor Ab. Detergent extract of ConA-activated D.10 cells was resolved by SDS-PAGE and subjected to electrophoretic transfer and immunochemical staining for Ubi-L receptor. As shown in Fig. 5A, a major protein band at 82 kDa and a minor band at 65 kDa under nonreducing conditions were detected by the anti-receptor Ab (lane 2). This finding indicates sequence homology between 82-kDa and 65-kDa proteins. We speculate that a protease, for instance, endoproteinase, has cleaved up to 17 kDa from the intact 82-kDa receptor, which results in the generation of receptor fragment that has lost the ability to bind Ubi-L but not the capacity to interact with the anti-receptor Ab. The migrated positions of these bands were unchanged under reducing conditions (lane 3). The same results were obtained from antigen (conalbumin)-stimulated D.10 cells (not shown). In contrast, the Ubi-L receptor was not seen in detergent extract of unstimulated D.10 cells (lane 4). We next performed deglycosylation analysis of Ubi-L receptor proteins because the 82-kDa protein may be glycosylated (Fig. 4C). The membrane fraction of ConA-activated D.10 cells was treated with neuraminidase and subjected to SDS-PAGE and immunoblotting as described above. Deglycosylation converted 82-kDa and 65-kDa molecules to 74-kDa and 60-kDa molecules, respectively (Fig. 5B). The mobility on a gel was changed further by additional treatment with N-glycanase, albeit a product was very faint (data not shown). These findings indicate that Ubi-L receptor protein may be a glycoprotein.

Immunoprecipitation of Ubi-L Receptor Complex—We next determined whether antisera prepared against the 82-kDa protein band would immunoprecipitate the cross-linked complex of 125I-Ubi-L (8 kDa) and its receptor. A band of 90 kDa was obtained (Fig. 6A), although several bands including 98-, 106-, 114-kDa were also observed presumably because of self-aggregation of 125I-Ubi-L. Control serum did not immunoprecipitate any complexes. To investigate whether proteins other than the 82-kDa polypeptide are associated with Ubi-L receptor complex, extracts of biotinylated D.10 cells were immunoprecipitated with anti-Ubi-L receptor Ab. As can be seen in Fig. 6B, the 105-kDa protein was coimmunoprecipitated by the anti-receptor (82-kDa polypeptide) Ab. We speculate that this 105-kDa protein may be involved in the signal transduction of Ubi-L, although it lacks an ability to bind Ubi-L itself.

Neutralization of Ubi-L Activity by Anti-Ubi-L Receptor Ab—To demonstrate that the 82-kDa protein is involved in Ubi-L-mediated signal transduction, a neutralizing test was carried out by the use of specific Ab (IgG) to this protein. As shown in Table II, this Ab could neutralize the Ubi-L activity, confirming that the 82-kDa protein is bioactive Ubi-L receptor. Interestingly, this Ab abolished the synergism between Ubi-L and IFN-γ. This finding supports the notion that IFN-γ might enhance the expression of Ubi-L receptor protein on the target cells (27).

Determination of the Amino Acid Sequence of Ubi-L Receptor Protein—We next attempted to analyze the amino acid sequence of the 82-kDa Ubi-L receptor protein (Table III). The NH2-terminal amino acid sequence of the receptor protein could not be determined probably because of acetylation. Thus this protein was digested with trypsin, separated by reverse phase HPLC, and tryptic peptides were sequenced. As shown below, four of the five peptide sequences derived from Ubi-L receptor are in alignment with a related sequence found in the
open reading frame predicted by the DNA sequence of the cDNA encoding mouse IL-11 receptor (28). The similarity of Ubi-L receptor peptide sequences to sequences in mouse IL-11 receptor as well as the similarity in size of the two proteins suggests that the Ubi-L receptor might be an another closely related protein. It should be noted that tryptic peptide 2 contains a WSXWS motif commonly seen in a cytokine receptor family (29).

**TABLE III**

**Alignment between amino acid sequence derived from sequencing Ubi-L receptor (R) and the corresponding sequences from the open reading frame of cloned marine IL-11 receptor**

| Factors | Antibody (100 μg/ml)* | Inhibition a |
|---------|-----------------------|-------------|
| Ubi-L (5 units/ml) | - | 26 ± 2 |
| IFN-γ (0.5 unit/ml) | - | 15 ± 2 |
| Ubi-L (5 units/ml) | - | 96 ± 4 |
| IFN-γ (0.5 unit/ml) | - | 8 ± 4 |
| Ubi-L (100 units/ml) | - | 80 ± 9 |
| IFN-γ (20 units/ml) | - | 95 ± 8 |
| - | - | 89 ± 6 |

* Specific antibody was added on day 0.

a Inhibition (%) of IgE production was calculated by comparison with the control response (968 ± 64 ng/ml). The results are the means ± S.D. of three separate experiments.

**FIG. 6. Immunoprecipitation by specific Ab to Ubi-L receptor protein. Panel A, immunoprecipitation of 125I-Ubi-L receptor complex by anti-82-kDa protein Ab. 125I-Ubi-L cross-linked to the purified receptor was immunoprecipitated with Ab (IgG) and analyzed by SDS-PAGE followed by autoradiography. Lane 1, IgG to the 82-kDa band; lane 2, IgG to complete Freund’s adjuvant. The positions of molecular mass markers (kDa) are shown on the left. Panel B, immunoprecipitation of biotinylated D.10 cells by anti-82-kDa protein Ab. Extracts of biotinylated D.10 cells were immunoprecipitated by the Ab and analyzed by SDS-PAGE followed by the ECL system. Lane 1, unstimulated D.10 cells; lanes 2 and 3, ConA-stimulated (48 h); lanes 1 and 2, anti-82-kDa protein IgG; lane 3, control IgG.

**TABLE II**

**Abrogation of Ubi-L activity by specific antibody to its receptor**

B cells were prepared and cultured as described under “Experimental Procedures.”

| Factors | Antibody (100 μg/ml)* | Inhibition a |
|---------|-----------------------|-------------|
| Ubi-L (5 units/ml) | - | 26 ± 2 |
| IFN-γ (0.5 unit/ml) | - | 15 ± 2 |
| Ubi-L (5 units/ml) | - | 96 ± 4 |
| IFN-γ (0.5 unit/ml) | - | 8 ± 4 |
| Ubi-L (100 units/ml) | - | 80 ± 9 |
| IFN-γ (20 units/ml) | - | 95 ± 8 |
| - | - | 89 ± 6 |

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a Inhibition (%) of IgE production was calculated by comparison with the control response (968 ± 64 ng/ml). The results are the means ± S.D. of three separate experiments.

**DISCUSSION**

Several lines of evidence have emerged from our studies indicating that a ubiquitin-like polypeptide specifically binds to target cells such as D.10 cells. We employed D.10 cells throughout the experiments because the response to Ubi-L had been well characterized in our previous study (25). The binding of Ubi-L to its receptor might be specific for the following reasons. (i) Ubi-L bound to antigen-stimulated but not to unstimulated D.10 cells. (ii) The binding of bio-Ubi-L was inhibited by the addition of unlabeled Ubi-L but not by irrelevant proteins. (iii) The binding showed species specificity. (iv) The molecular mass of affinity-purified Ubi-L receptor was the same as that of the protein obtained from ligand blot assay. (v) The antigenicity of these proteins was much the same. Finally (vi) Ubi-L activity was neutralized by anti-Ubi-L receptor Ab. On SDS-PAGE under nonreducing conditions, the apparent molecular weight of the Ubi-L receptor was 82,000. This apparent size was unchanged by the disulfide reducing agent dithiothreitol, indicating a lack of disulfide bonds to other proteins. This 82-kDa protein may be associated with the 105-kDa protein lacking an ability to bind Ubi-L (Fig. 6B). Characterization of this 105-kDa protein is under way to clarify its function. Scatchard analysis of Ubi-L receptor was unsuccessful because of an aggregative property of recombinant Ubi-L. Of note, Ubi-L has a secondary structure containing the tandem largely hydrophobic structural units. This likely accounts for our consistently poor yield of Ubi-L after cleavage from the fusion partner GST. It is difficult to handle a small amount of Ubi-L even in the presence of a carrier protein such as bovine serum albumin. In contrast, ubiquitin has a stable secondary structure containing only a low percentage of α-helix or β-sheet (30).

It has been claimed that suppressor factors consist of effector and accessory molecules (31). Like antigen-specific suppressors, hybridoma-derived (native) 70-kDa MNSF consists of 8-kDa Ubi-L (MNSF-β) and 62-kDa MNSF-α, a polypeptide serologically related to TCR-α chain (17). We speculate that the Ubi-L may possibly be a candidate for an effector molecule. Support for this theory is the fact that the Ubi-L itself bound directly to the target cells. On the other hand, MNSF-α is necessary for the extracellular release and stability of Ubi-L. Whether MNSF-α might contribute to the Ubi-L binding to its target cells remains to be determined. To date, isolation of cDNA encoding MNSF-α has been unsuccessful because of a transient and faint expression of the mRNA.

It is evident that several cell surface receptor proteins are ubiquitinated. For instance, platelet-derived growth factor receptor is a ubiquitin acceptor (8, 9). The intracellular domain of
this receptor is ubiquitinated, which leads to intracellular signaling. Similarly, cytosolic regions of the TNF-α receptor (32), ζ-subunit of the TCR-CD3 complex (6), and high affinity IgE receptor (7) are ubiquitinated. Interestingly, Ab specific for ubiquitin detected antigenic determinants on the cells expressing lymphocyte homing receptor, indicating that ubiquitin covalently binds to the extracellular domain of this receptor (33, 34). Although Ubi-L also recognizes cell surface receptor proteins, it seems unlikely that it binds covalently to the receptors in a manner similar to ubiquitination because MNSF-β, which lacks the free COOH-terminal glycol doublet responsible for isopeptide bond formation, bound to target cells (Fig. 1).

Ubi-L is not only the molecular tag for protein modification. Sentrin (18) and UCRP (19) have been shown to be conjugated to other proteins in a process analogous to ubiquitination. The COOH termini of UCRP and sentrin are processed efficiently, which allows for subsequent protein conjugation. Similarly, Ubi-L is cleaved from a fusion partner, ribosomal protein S30, in cytosol. Like UCRP, Ubi-L conjugates to intracellular proteins in vitro (15) and in vivo (16). Ubi-L conjugates to intracellular acceptor proteins including MNSF-α. The Ubi-L conjugation is similar but not identical to the ubiquitination process because acceptor proteins for Ubi-L are different from those for ubiquitin (15). The Ubi-L conjugation is thought to occur via isopeptide bond formation because isopeptidase prepared from murine livers dissociates MNSF-α from Ubi-L (17). In this context, we speculate that the intracellular and extracellular mode of actions of Ubi-L may differ.

A number of biotinylated cytokines have been employed for receptor analysis instead of iodination (35–37). Bio-Ubi-L shows biological activity similar to that of unlabeled Ubi-L. Nevertheless, cross-linking experiments with the use of biotinylated or iodinated Ubi-L were unsuccessful because of the poor compatibility of the biotinylated or iodinated proteins with murine cell cultures. Nevertheless, cross-linking experiments with the use of biotinylated cytokines have been employed for receptor analysis instead of iodination (35–37). Bio-Ubi-L shows biological activity similar to that of unlabeled Ubi-L. Nevertheless, cross-linking experiments with the use of biotinylated or iodinated Ubi-L were unsuccessful because of the poor compatibility of the biotinylated or iodinated proteins with murine cell cultures.