Association of intercellular adhesion molecule 1 with the multichain high-affinity interleukin 2 receptor

(lymphocyte function-associated antigen 1/interleukin 2 receptor α subunit)

J. Burton*, C. K. Goldman*, P. Rao†, M. Moos‡, and T. A. Waldmann*

*Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; †Center for Biologies Evaluation and Research Division of Biochemistry and Biophysics, Bureau of Biologics, Bethesda, MD 20892; ‡Immunology Division, Ortho Pharmaceutical Corporation, Raritan, NJ 08869

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ABSTRACT Previously, using flow cytometric resonance energy transfer and lateral diffusion measurements, we demonstrated that a 95-kDa protein identified by two monoclonal antibodies (OKT27 and OKT27b) interacts physically with the 55-kDa α protein of the high-affinity interleukin 2 (IL-2) receptor. In the present study, this 95-kDa protein (p95) was purified and amino acid sequence data were obtained that showed strong homology to the human intercellular adhesion molecule 1 (ICAM-1). The identity of the p95 protein with ICAM-1 was confirmed by sequential immunoprecipitations using OKT27 and an antibody, WEHI-CAM-1, that is directed toward ICAM-1. We confirmed the physical proximity of p95/ICAM-1 to the IL-2 receptor α subunit by demonstrating that radiolabeled IL-2 could be cross-linked to this protein expressed on activated T cells. In functional studies, the antibodies OKT27 and OKT27b inhibited T-cell proliferative responses to OKT3, to soluble antigen, and to heterologous cells (mixed lymphocyte reaction). However, these antibodies did not inhibit IL-2-induced proliferation of an IL-2-dependent T-cell line. Taken together with our previous observations, the present studies suggest that ICAM-1 is in proximity and interacts physically with the high-affinity IL-2 receptor. The association of ICAM-1 with the IL-2 receptor may facilitate the paracrine IL-2-mediated stimulation of T cells expressing IL-2 receptors by augmenting homotypic T–T-cell interaction, by receptor-directed focusing of IL-2 release by helper T cells, and by focusing IL-2 receptors of the physically linked cells to the site of lymphocyte function-associated antigen 1–ICAM-1–IL-2 receptor interaction.

T lymphocytes stimulated with antigen produce interleukin 2 (IL-2) (1, 2). The hormone exerts its biological effect through specific high-affinity receptors expressed on activated but not resting T cells (3). There are two classes of IL-2 receptors (IL-2R) differing 1000-fold in their affinities for IL-2 (4). Both high-affinity (Kd = 10 pm) and low-affinity (Kd = 10 nM) receptors share the 55-kDa α subunit protein (p55 or IL-2Ra) defined by the anti-Tac monoclonal antibody (4–6). By using cross-linking methodology and radiolabeled IL-2, we and others have identified an additional non-Tac IL-2 binding protein of 70/75 kDa (p75 or IL-2Rβ chain) (6, 7). Evidence has been reported suggesting a more complex subunit structure that involves proteins in addition to the p55 and p75 IL-2 binding chains (4, 8–12). In one series of studies, we used two monoclonal antibodies, OKT27 and OKT27b, that were shown to react with distinct epitopes of a 95-kDa protein (p95) (8, 9). There was a low level of p95 expression on resting T cells that increased on T-cell activation in parallel with induced IL-2Ra expression (8). The OKT27b antibody occasionally coprecipitated a 55-kDa protein as well as the 95-kDa protein from radiolabeled cells expressing the high-affinity IL-2R. The relationship between p95 identified by OKT27 and p55 identified by the anti-Tac monoclonal antibody was investigated in intact cells by labeling each protein with the appropriate fluorescent monoclonal antibody and then measuring the average distance between them with a flow cytometric fluorescence energy transfer technique. This technique permits the determination of intermolecular distances of 2–10 nm on a cell-by-cell basis. The energy transfer data showed proximity of the p95 and p55 proteins and indicated that p55 and p95 proteins are associated on the surface of the activated T cells examined (8). Furthermore, in additional studies we used recovery from photobleaching measurements to support the view that p55 and p95 interact physically in situ in cells expressing high-affinity IL-2R (9). Specifically, p95 was immobilized by a monoclonal antibody (OKT27) directed against it. Furthermore, this treatment with OKT27 also affected the lateral diffusion of the IL-2Ra chain so that this protein became immobilized in >30% of the cells pretreated with OKT27 (9). These findings extended the results from immunoprecipitation and energy transfer experiments and indicated that not only are p95 and p55 in proximity but they interact physically on cells expressing both molecules.

The present studies were directed toward purification and characterization of the p95 protein associated with the IL-2R. Furthermore, we wished to define the role played by p95 in cell–cell interactions and in cellular signaling.

MATERIALS AND METHODS

Monoclonal Antibodies. The production and specificities of the monoclonal antibodies anti-Tac (directed to the 55-kDa α subunit of the IL-2R) and OKT27 and OKT27b (directed toward a 95-kDa cell membrane protein) have been described (5, 8). RPC5 and UPC10 are IgG2a murine myeloma proteins that do not bind to human cells. WEHI-CAM-1, an anti-CD54 antibody identifying intercellular adhesion molecule 1 (ICAM-1) was generously provided by A. W. Boyd (Walter and Eliza Hall Institute, Melbourne, Australia) (13). CL203.4 is an anti-CD54 antibody generously provided by S. Ferrone (New York Medical College, Valhalla, NY).

Cell Lines. The HuT 102B2 cell line expresses high-affinity IL-2R. Kit 225, an IL-2-dependent T-cell line expressing the high-affinity IL-2R was kindly provided by Takashi Uchiuma (Kyoto University, Kyoto, Japan) (14). The YT cell line obtained from Junji Yodoi (Kyoto University, Kyoto, Japan) is an IL-2-independent cell line that expresses predominantly the IL-2Rβ chain with a low level of expression of the IL-2Ra chain (15). The cell lines were maintained in RPMI 1640

Abbreviations: ICAM-1, intercellular adhesion molecule-1 (CD54); LFA-1, lymphocyte function-associated antigen-1; IL-2, interleukin 2; IL-2Ra/β, IL-2 receptor α/β; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosylphenylalanil chloromethyl ketone; PBMC, peripheral blood mononuclear cell.
medium supplemented with 10% fetal calf serum. In the case of Kit 225, this was supplemented with 0.5 nM recombinant IL-2.

**Purification of p95 Identified by OKT27.** OKT27 and control UPC10 immunoaffinity columns were prepared by cross-linking these purified monoclonal antibodies to protein A agarose (Bio-Rad) with dimethyl pimelimidate (Pierce) according to the method of Schneider et al. (16). Approximately 2 x 10^9 HuT 102B2 cells were washed three times in Dulbecco’s phosphate-buffered saline without calcium or magnesium (D-PBS) and then suspended in 200 ml of extraction buffer (10 mM Tris-HCl, pH 7.4/0.2 mM MgCl_2/0.2 mM CaCl_2/1 mM phenylmethylsulfonyl fluoride (PMSF)/0.25 M sucrose/antipain (1.5 μg/ml)/0.2 mM tosylphenylalanyl chloromethyl ketone (TPCK))/0.7% Sarkosyl, (ii) 100/0.7% Triton X-100, (iii) 10/0.1% SDS/12.5% polyacrylamide gel containing 0.1% SDS, electroeluted according to the method of Laemmli (19), and analyzed by autoradiography.

**IL-2 Binding Assay.** The binding of radioiodinated IL-2 to cells was measured as described by Robb et al. (20). Non-specific binding was determined in the presence of a 200-fold excess of unlabeled IL-2. Specific binding was obtained by subtracting non-specific from total binding.

**T-Cell Proliferation.** Human peripheral blood mononuclear cells (PBMCs) were isolated from normal volunteers by Ficoll/Hypaque density-gradient centrifugation. One hundred thousand cells in 0.2 ml of Ex Vivo 10 serum-free defined medium (Whittaker M.A. Bioproducts) were placed into 96-well plates (Costar); 2 ng of purified OKT3 per ml (Ortho Diagnostics) was added to all but the background wells to activate T cells. In parallel studies, PBMCs were cultured with tetanus antigen (Massachusetts Department of Health). Finally, mixed leukocyte cultures were prepared in which 1 x 10^5 PBMCs in 200 μl of RPMI 1640 medium containing 10% pooled AB serum (Sigma) were cultured with irradiated (2000 cGy at 1800 cGy/min from a 137Cs source) control allogeneic PBMCs. The cells were cultured for 96 hr with a terminal 16-hr addition of 1 μCi of [[H]thymidine per well (45 Ci/mmol; 1 Ci = 37 GBq; Amersham). At the end of the culture period, the cells were harvested with a multisample harvester (Skatron, Sterling, VA) and assayed in a liquid scintillation counter. The cell cultures were studied in triplicate.

**IL-2-Dependent Proliferation.** The human IL-2-dependent T-cell line Kit 225 expressing high-affinity IL-2R was used. The cells were cultured in medium supplemented with 0.5 nM recombinant IL-2 (specific activity, 3 x 10^6 units/mg; Cetus). Before each assay, the Kit 225 cells were cultured in RPMI 1640 medium with 10% fetal calf serum without IL-2 for 3–4 days. Cells were added to 96-well plates at 2.5 x 10^4 cells per well in a final vol of 200 μl. Recombinant IL-2 (Cetus) was added to the wells to yield concentrations ranging from 0.78 to 200 pM. Assays were performed in the absence and in the presence of monoclonal antibodies (20 μg/ml) to determine their capacity to inhibit IL-2-dependent proliferation. The culture period was 72 hr with a terminal 16-hr pulse of 1 μCi of [[H]thymidine per well as described above.

**RESULTS**

On the basis of previous studies using fluorescence energy transfer and lateral diffusion measurements, we concluded that a 95-kDa protein identified by the two monoclonal antibodies OKT27 and OKT27b is associated and interacts physically with the p55 protein of the high-affinity IL-2R. The studies we now describe were initially directed toward purification and characterization of the p95 protein using OKT27 antibody affinity chromatography. Solubilized cell membrane lysates were prepared from 2 x 10^10 HuT 102B2 cells. The resulting membrane lysate was passed twice through a nonspecific UPC10 agarose column. The effluent from this column was then applied to an OKT27 agarose column, which was eluted with 2.5% acetic acid. The eluate was lyophilized and redissolved in a small volume of 2.5% acetic acid for further analysis.

**Cross-Linking Study.** 125I-labeled IL-2 was chemically cross-linked by using sulfo-N-succinimidyl 4-(p-maleimidophenyl)butyrate at a 1 mM final concentration with intact HuT 102B2 cells as described (6). Cells were then solubilized with D-PBS/1% Triton X-100/PMSF/TPCK using 0.6 ml of buffer per 10^6 cells.

**Immunoprecipitation.** Viable whole HuT 102B2 cells were radioiodinated by a modification of the lactoperoxidase technique. Cells labeled with radiiodine by this procedure were washed twice and then solubilized and immunoprecipitated with OKT27 or WEHI-CAM-1 by the method of Goldman and Liu (18). Fixed *Staphylococcus aureus* Cowan strain I precipitates were incubated in sample buffer at 100°C for 3 min to elute bound material. These eluates were applied to a SDS/12.5% polyacrylamide gel containing 0.1% SDS, electrophoresed according to the method of Laemmli (19), and analyzed by autoradiography.
amide gel.

125I-labeled IL-2 (4 nM) was incubated with HuT 102B2 cells followed by covalent cross-linking as described. Triton X-100 solubilized whole cell extracts were passed through an OKT27 agarose column and eluted with 2.5% acetic acid. This eluate was then run on a Laemmli SDS/8.5% polyacrylamide gel and analyzed by autoradiography. The numbers on the right are molecular mass markers in kDa. The numbers on the left represent the estimated molecular mass of the relevant bands in kDa.

In experiments reported previously, we demonstrated a physical proximity and interaction of p95 with the α chain of the IL-2R. We wished to examine further the physical relationship of p95/ICAM-1 to the IL-2R. We therefore sought to determine whether radiolabeled IL-2 became cross-linked to p95/ICAM-1 by dint of its proximity to IL-2Ra. For these studies, 125I-labeled IL-2 was cross-linked to HuT 102B2 cells by using sulfosuccinimidyl 4(4-p-maleimidophenyl)butyrate. A series of radiolabeled bands were observed when an aliquot of 125I-labeled IL-2 cross-linked detergent-solubilized HuT 102B2 cells was analyzed on a SDS/polyacrylamide gel, including bands at ~110 kDa that might reflect the p95 protein associated with the 15-kDa IL-2. To define whether this band represented p95/ICAM-1 cross-linked to radiolabeled IL-2, 125I-labeled IL-2 cross-linked HuT 102B2 cell membranes were solubilized and passed through an OKT27 affinity column. The column eluates were examined by SDS/PAGE. The majority of the radiolabeled material at 110 kDa did not bind to the OKT27 column and probably reflects the 95- to 105-kDa non-ICAM-1 protein that has previously been reported to be associated with the IL-2R (10, 11). Nevertheless, by this approach, a small proportion of the radiolabel was found to be cross-linked to p95/ICAM-1 and ran as a doublet with molecular masses of approximately 120 and 150 kDa (Fig. 2). The presence of the 120-kDa size range is consistent with the presence of IL-2 ligand covalently cross-linked to p95. The presence of the larger band is consistent with IL-2 cross-linked to a 135-kDa band that had been observed in some early affinity purifications.

We felt that there existed two alternative explanations for the binding of radiolabeled IL-2 to p95/ICAM-1. First, it was conceivable although unlikely that p95 represents a bona fide IL-2 binding protein. Alternatively, p95 may not have intrinsic IL-2 binding capacity but it may become cross-linked to radiolabeled IL-2 because of its proximity to the IL-2Ra or β chains. To address this issue, HuT 102B2 and the YT cell line were used in 125I-labeled IL-2 binding experiments in the presence and absence of appropriate monoclonal antibodies at 50 μg/ml. As shown in Fig. 3, OKT7 did not block the binding of 125I-labeled IL-2 to the HuT 102B2 cell line. Similarly, OKT27b and another anti-ICAM-1 antibody, CL03.4, both of which recognize epitopes distinct from that.

**Fig. 1.** Characteristics of proteins precipitated by OKT27 and WEHI-CAM-1. Triton X-100 extracts of HuT 102B2 cells labeled with 125I were incubated with the monoclonal antibodies indicated, immunoprecipitated with S. aureus cells, and then electrophoresed under reducing conditions through a Laemmli SDS/12.5% polyacrylamide gel. In the case indicated, the extract was precleared with WEHI-CAM-1 and the supernatants were then immunoprecipitated with OKT24 and electrophoresed as indicated above. The number on the left is the molecular mass of the relevant band in kDa.

**Fig. 2.** 125I-labeled IL-2 (4 nM) was incubated with HuT 102B2 cells followed by covalent cross-linking as described. Triton X-100 solubilized whole cell extracts were passed through an OKT27 agarose column and eluted with 2.5% acetic acid. This eluate was then run on a Laemmli SDS/8.5% polyacrylamide gel and analyzed by autoradiography. The numbers on the right are molecular mass markers in kDa. The numbers on the left represent the estimated molecular mass of the relevant bands in kDa.

**Fig. 3.** Scatchard analysis of one of several 125I-labeled IL-2 binding studies performed on the HuT 102B2 cell line. All studies resulted in nearly identical curves. 125I-labeled IL-2 binding was done in the presence of the monoclonal antibody anti-Tac (a) or OKT27 (c) at 50 μg/ml or with medium alone (●).
of OKT27, did not inhibit IL-2 binding. In contrast, anti-Tac,
an antibody to IL-2Rα, abrogated radiolabeled IL-2 binding
to this cell line. In parallel studies, neither OKT27 nor
OKT27b affected radiolabeled IL-2 binding to the YT cell
line. These results support the view that p95/ICAM-1 is
associated with the IL-2Rα chain in the high-affinity IL-2R
but is not a protein with inherent IL-2 binding capacity.

Functional immunological experiments were carried out to
define the role that might be played by p95/ICAM-1 in the
process of lymphocyte activation and differentiation. Since
anti-ICAM-1 antibodies have been reported to affect various
lymphocyte proliferation assays (13, 24), we defined the
effect of the OKT27 and OKT27b monoclonal antibodies
used in these studies on OKT3-induced proliferation. Dose-
dependent inhibition of the proliferation of peripheral blood
mononuclear cells induced by OKT3 (2 ng/ml) was observed
with both OKT27 and OKT27b antibodies with more pro-
nounced effects being seen with the OKT27 antibody (Fig. 4).
Similar inhibitory effects were also observed with tetanus
antigen as well as with mixed leukocyte-induced proliferation
(data not shown).

Proliferations induced by OKT3, antigen, or the mixed
leukocyte reaction are complex multistep events. An effective
proliferative response requires the direct interaction and
adhesion of different cell populations leading to induction of
the expression of IL-2 and its receptor. The final proliferative
events require the interaction of IL-2 with the high-affinity
multisubunit IL-2R. To try to differentiate among the many
potential sites of action of OKT27 and OKT27b on lympho-
cyte proliferation, we chose an assay system in which prolif-
eration was due solely to the binding of IL-2 to its receptor.
Specifically, we used the human IL-2-dependent T-cell line
Kit 225, which expresses the high-affinity IL-2R as well as
large numbers of the p95/ICAM-1. This cell line manifests a
dose-dependent proliferative response to IL-2 that is pro-
foundly inhibited by the addition of the anti-Tac monoclonal
antibody. However, as shown in Fig. 5, there was no inhibitory
effect on IL-2-induced proliferation observed with OKT27 or
with the other anti-ICAM-1 antibodies tested. Furthermore,
the addition of these antibodies did not increase the effect
mediated by anti-Tac. Thus, the inhibition of lymphocyte
proliferation, in response to mitogen or antigen,
induced by OKT27, OKT27b, and other anti-ICAM-1
antibodies appears to be due to mechanisms other than an
inhibition of IL-2/IL-2R-mediated signal transduction when
IL-2 is presented to the cells in medium in which cell–cell
interaction is not required. As noted below, this observation
does not exclude the possibility that ICAM-1/lymphocyte
function associated antigen 1 (LFA-1) expression on acti-
tivated T cells facilitates T–T cell interaction leading to recep-
tor-directed focusing of IL-2 release by helper T cells with
subsequent ligand binding by physically linked T cells that
express focused associated IL-2R–ICAM-1 proteins.

**DISCUSSION**

The high-affinity receptor for IL-2 is a complex structure
that involves proteins in addition to the p55 and p75 IL-2 binding
proteins. With the use of coprecipitation analysis, radiola-
beled IL-2 cross-linking procedures, and flow cytometric
resonance energy transfer measurements, a series of addi-
tional proteins of 22, 35, 40, 75 (non-IL-2 binding), and
95–105 kDa have been associated with the two IL-2 binding
chains (4, 8–11, 25). Furthermore, an association between
IL-2R and class I HLA molecules on T cells has been
demonstrated (12). Previously we used flow cytometric res-
onance energy transfer and fluorescent photobleaching
recovery measurements to demonstrate the association of
the IL-2Rα with a 95-kDa protein identified with the two mono-
clonal antibodies OKT27 and OKT27b. In the current study,
we present evidence suggesting that the p95 protein associ-
ated with the IL-2R is ICAM-1 (CD54). The amino acid
sequence of the protein purified from HuT 102B2 cell lines
using OKT27 showed homology to ICAM-1 and to no other
protein in the data bases searched. The identity of p95 with
ICAM-1 was further supported by the observations using
preclearing immunoprecipitation analysis. OKT27 pre-
cleared most of the 95-kDa antigen recognized by the estab-
lished anti-ICAM-1 antibody WEHI-ICAM-1. In addition,
while the current studies were under way, the antibodies
OKT27 and OKT27b were grouped in the CD54 (ICAM-1)
class at the 4th International Workshop and Conference on
Human Leukocyte Differentiation Antigens (26).

ICAM-1 (CD54) binds to the β2-integrin, LFA-1 (CD11a/
CD18), promoting cell adhesion in immune inflammatory
reactions (27–29). The effective interaction between T cells
and other cells requires that recognition of a specific antigen
be coordinated with cell adhesion. The interaction of the
LFA-1–ICAM-1 receptor ligand pair is critical for a series of
T-cell functions. The adhesion mediated by T-cell LFA-1
interacting with ICAM-1 allows T cells to interact with
antigen-presenting cells and thereby initiate an immune
response. In addition, T-cell LFA-1 binding to target cell
ICAM-1 can be important in the tight adhesion between
cytotoxic T cells and their targets. The present studies taken
ontogether with our previous reports show an association and
physical interaction between ICAM-1 and the IL-2Rα. In
many cases, antibodies directed toward the IL-2Rα and
toward the ICAM–LFA-1 antigen receptor and ligand pair
have similar functional effects. Both sets of antibodies inhibit
antigen and mixed leukocyte-induced proliferation and the
generation of cytotoxic T cells and inhibit T-cell-dependent
antibody synthesis. Furthermore, both sets of antibodies
have been used successfully to inhibit allograft rejection
(30–32).

Using OKT27 and OKT27b monoclonal antibodies, we
confirmed that anti-ICAM-1 antibodies inhibit OKT3, anti-
gen, and mixed leukocyte-induced T-cell proliferation. This
presumably reflects in part the requirement for T-cell acti-
vation of cell contact between T cells and antigen-presenting
cells. However, there was no inhibitory effect with either
OKT27 or OKT27b on IL-2-induced proliferation by an
IL-2-dependent T-cell line. This parallels the previous ob-
In further support of this model, Poo et al. (35) have demonstrated receptor-directed focusing of lymphokine release by helper T cells. Using a porous membrane and a cloned murine helper T-cell line, they showed that lymphokine release occurs preferentially over the area of receptor cross-linking under conditions of limited helper T-cell activation. Furthermore, the interaction of ICAM-1 with LFA-1 may focus the IL-2R to this site of cell-cell interaction and focused lymphokine release. In this regard, previous studies using recovery from photobleaching demonstrated that the lateral diffusion of the IL-2Ra, normally a very mobile cell-surface molecule, was significantly reduced in the presence of the OKT27 antibody to ICAM-1 (9). The interaction of LFA-1 with ICAM-1 may produce a similar immobilization of the ICAM-1-IL-2R complex, thereby focusing IL-2R to the site of membrane interaction and IL-2 release.

Finally, the interaction of IL-2 with a high-affinity receptor leads to the modulation of this receptor from the cell surface. Similar to the interaction of IL-2 with cells expressing high-affinity IL-2R and ICAM-1 down-regulates cell-surface ICAM-1 expression (36). This may reflect modulation of ICAM-1 with the associated high-affinity IL-2R. This IL-2-mediated down-regulation of ICAM-1 expression may facilitate deadhesion of the cells following IL-2-IL-2R interaction. In conclusion, we have demonstrated that p95 defined by OKT27 and OKT27b is ICAM-1. Taken together with our previous observations, this suggests that ICAM-1 is in proximity and interacts physically with the high-affinity IL-2R on cells expressing both molecules. The association of ICAM-1 with the IL-2R may facilitate the paracrine IL-2-mediated stimulation of T cells expressing IL-2R by augmenting homotypic T-T-cell interaction, by receptor-directed focusing of IL-2 release by the helper T cell, and by focusing IL-2R of the physically linked cells to this site of LFA-1-ICAM-1-IL-2R interaction.