Affinity cross-linking of membrane bound $^{125}$I-interleukin-6 (IL-6) on several cell lines revealed a three-band pattern of IL-6-containing cross-linked complexes with molecular masses of 100, 120, and 150 kDa. To identify the membrane components that were associated with IL-6 in the three complexes, we employed the Denny-Jaffe reagent, a heterobifunctional, cleavable cross-linker that allows the transfer of $^{125}$I from the ligand to its receptor. Samples cross-linked with Denny-Jaffe reagent were analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis in which the cross-linker was cleaved prior to the second dimension. This analysis revealed that IL-6 directly associates with a 130-kDa membrane protein thus allowing the formation of the 150-kDa complex. In addition, both the 100- and 120-kDa cross-linked complexes were shown to include an 80-kDa membrane glycoprotein associated with one and two IL-6 molecules, respectively.

Interleukin 6 (IL-6) is a cytokine that displays a broad range of biologic activities (reviewed in Refs. (1) and (2)) including the stimulation of immunoglobulin production by activated B cells (3), the support of myeloma (4, 5), plasmacytoma, and hybridoma proliferation (6, 7), and the induction of acute phase responses in hepatocytes (8). IL-6 appears to mediate its effects by interacting with specific receptors on the cell surface. Binding studies have revealed the presence of both low affinity (12, 13) and high affinity (14, 15) binding sites for IL-6 on a variety of cells. The cDNAs of two transmembrane glycoproteins that participate in the IL-6 receptor system, gp80 and gp130, with molecular masses of 80 and 130 kDa, have been cloned, sequenced, and expressed in eukaryotic cells (14, 15). The gp80 receptor molecule directly binds IL-6 with low affinity (15) and has been shown to associate with gp130 in the presence of IL-6 (16). Antibodies to gp130 inhibit the formation of high affinity binding sites for IL-6 thus indicating that gp130 participates in the formation of high affinity binding sites (14). Studies have also shown that the combination of IL-6 plus a soluble form of recombinant gp80, that lacks transmembrane and intracellular domains, can associate with gp130 to initiate IL-6 responses (16). This result implicates gp130 as a signal transducer in the generation of IL-6 responses (14, 16) and indicates that the transmembrane and intracellular portions of gp80 are not necessary for signal transduction. Although gp130 participates in the formation of high affinity binding sites for IL-6 and in the generation of IL-6 responses, gp130 has thus far not been found to bind or directly interact with IL-6 itself. This has led to the hypothesis that gp130 is a nonligand binding member of the IL-6 receptor system and that only gp80 contributes to IL-6 binding (14, 16).

Although only gp80 has been documented to be an IL-6-binding molecule, several studies incorporating cell surface affinity cross-linking with $^{125}$I-IL-6 have reported the formation of multiple IL-6-containing complexes ranging from 100 to 200 kDa (12, 17, 18), thus suggesting that the IL-6 receptor complex may consist of more than a single IL-6-binding protein. In this paper, we describe the characterization of the IL-6 receptor system on the human myeloma cell line, U266, using both homobifunctional and heterobifunctional affinity cross-linking techniques. Our results demonstrate that IL-6 associates directly with a 130-kDa membrane glycoprotein. In addition, our studies also reveal that two IL-6 molecules can be found complexed with an 80-kDa member of the receptor complex.

**MATERIALS AND METHODS**

**Cell Culture**—All cell culture was performed in complete medium, which consisted of RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (Whittaker Bioproducts, Walkersville, MD) and antibiotics, at 37 °C in a humidified atmosphere of 7% CO$_2$. U266, a human myeloma cell line, and U937, a human histiocytic lymphoma cell line, were obtained from the American Type Culture Collection (Rockville, MD). H929, a human myeloma cell line, was generously provided by Dr. Adi Gazdar, National Cancer Institute, NIH, Bethesda, MD (19). Peripheral blood lymphocytes were prepared from the blood of normal donors using Ficoll fractionation and activated by culturing overnight in 10 μg/ml phytohemagglutinin. The IL-6-dependent murine cell lines B9 and T1165 were grown in complete medium supplemented with 50 μM 2-mercaptoethanol and recombinant IL-6.

**Radiolabeling of Recombinant Human Interleukin-6**—The biologic activity of the recombinant human IL-6 (R&D Systems, Minneapolis, MN) used in these studies was tested on two IL-6-dependent cell lines, B9 (20) and T1165 (6), and found to have $1 \times 10^9$ hybridoma growth factor units/mg and $3 \times 10^7$ plasmacytoma growth factor units/mg, respectively. The specific activity was essentially unchanged after iodiumation. IL-6 was labeled using the $^{125}$I-diodo-Bolton-Hunter reagent (2200 Ci/mmol, Du Pont-New England Nuclear) according to the manufacturer's recommended procedure (21). Briefly, 2 μg of IL-6 in 15 μl of 33 mM sodium borate, 250 mM NaCl,
pH 8.5, were combined with 0.5 or 1.0 mCi of evaporated #.#iodo-
XO%, a reagent by size exclusion chromatography on a Bio-Gel
P-6 (Bio-
Rad) column previously coated with 50 mM Tris/HC1, pH 6.8, and 0.1% SDS. The reagent was then washed twice with phosphate-buffered saline to remove un-
bound bovine serum albumin. The recovery of labeled IL-6 was 75-
80%, and based on this value the specific activity of the #.1#-IL-6 was calculated to be 2.4 x 10^16 cpmm/mol. More than 99% of the
radioactivity corresponded to a single hand of IL-6 when analyzed
under reducing or nonreducing conditions according to Laemmli
(23). Commerically prepared #.1#-IL-6 (Du Pont-New
England Nuclear) was used in some experiments.

SDS-PAGE—Samples were analyzed by SDS-PAGE on 7% poly-
acrylamide slab gels (1.5 mm x 8 cm x 13 cm in the presence of
reducing or nonreducing conditions according to Laemmli (23). After
electrophoresis, gels were dried under vacuum prior to autoradiogra-
yphy.

Affinity Cross-linking of #.1#-IL-6 to Its Receptor—Affinity cross-
linking was performed using a modification of a previously published
procedue (24). Briefly, cells were washed twice and resuspended at 5
x 10^5 cells/ml in cold binding medium (RPMI 1640, 1.0% bovine serum
albumin, 0.05% sodium azide). Binding was allowed to proceed for 1
h on ice with the indicated amount of #.1#-IL-6. After binding, the
cells were washed with cold RPMI 1640, 0.05% sodium azide to
remove the unbound #.1#-IL-6 and resuspended in 1 ml of phosphate-
buffered saline. The pH of the #.1#-IL-6 was 8.5, and #.1#-IL-6 cross-linking was initiated by
the addition of 300 g/ml disuccinimidyl suberate, disuccinimidyl
tartrate, or dithiobis-(succinimidyl proprionate) (Pierce Chemical
Co.), as indicated, and allowed to proceed at 4 °C for 15 min. The
cross-linking reaction was stopped by centrifugation and by imme-
diate lysis of the cells with 50 mM Tris-HC1, 300 mM NaCl, 1%
Nonidet P-40, 1 mM phenylmethyisalfonyl fluoride, 10 mM levupentin,
and 10 mM pepstatin, pH 7.5 (lysis buffer), for 30 min on ice. The
lysat was centrifuged for 10 min at 15,000 x g and the supernatant
collected for immunoprecipitation (see below) or for direct analysis
by SDS-PAGE under reducing or nonreducing conditions.

Immunoprecipitation—CLB-IL-6/8, a murine monoclonal anti-IL-
6 antibody, and affinity-purified sheep anti-IL-6 polyclonal antibody
have been described elsewhere (25). Lysates obtained after affinity
cross-linking with #.1#-IL-6 were incubated overnight at 4 °C with
anti-IL-6 or control antibodies and precipitated with protein G-
Sepharose (Pharmacia LKB Biotechnology Inc.) for 4 h at 4 °C. The
precipitates were washed four times with lysis buffer and analyzed by
SDS-PAGE as indicated. In some experiments, antibodies conjugated
directly to Sepharose were used with similar results.

Glycosylation Studies—The contribution of glycosylation to the
molecular mass of the cross-linked receptor complexes was assessed
using a combination of: 1) inhibition of N-linked glycosylation by
2) subsequent cleavage of sialic acid and O-linked glycosidic residues.
Briefly, U266 cells, in the log phase of growth, were cultured for 18 h
at 5 x 10^6 cells/ml in complete medium containing 5 g/ml tunica-
mycin. The cells were harvested, cross-linked to #.1#-IL-6, immuno-
precipitated with sheep anti-IL-6, and immobilized on protein G-
Sepharose beads as described above. To remove sialic acid residues,
the immobilized immunoprecipitates were washed and resuspended in
0.1 M acetate buffer, pH 6.5, 10 mM CaCl2 containing 0.3 unit/ml
neuraminidase (Genzyme, Cambridge, MA) and incubated for 1 h at
37 °C. O-linked residues were then removed by resuspending the
immobilized immunoprecipitates in the same buffer containing 10
mM t-galactono-γ lactone plus 0.02 unit/ml O-glycanase (Genzyme)
and incubated overnight at 37 °C. The samples were analyzed by
SDS-PAGE and autoradiography.

Affinity Cross-linking with Denny-Jaffe Reactant—Recombinant
IL-6 (1 μg) was labeled with the Denny-Jaffe reactant (100 μCi, specific
activity 2200 Ci mmol, Du Pont-New England Nuclear) following
the procedure described above for the Bolton-Hunter re-
agent with the exception that the entire procedure was performed in
the dark. The specific activity was calculated to be 1.5 x 10^15 cpmm/
mol. For cross-linking experiments the cells were washed twice and
resuspended at 5 x 10^6 cells/ml in cold binding medium (RPMI 1640,
1% bovine serum albumin, 300 μg/ml bacitracin). Binding was allowed
to proceed in the dark for 1 h on ice with the indicated amount of
#.1#-Denny-Jaffe-IL-6. After binding, the cells were washed in the
dark with cold binding medium and resuspended in cold phosphate-
buffered saline. Photocross-linking was accomplished by exposing
samples for 15 min to 365 nm (long wave) UV light. The cells were
pelleted, resuspended in lysis buffer, and incubated for 30 min on ice.
The lysate was centrifuged for 5 min at 15,000 x g, and the super-
natant was immunoprecipitated with anti-IL-6 antibodies as indi-
cated and analyzed by one- and two-dimensional SDS-PAGE. For
two-dimensional analysis the immunoprecipitated lysate was applied
on a 7% SDS-PAGE tube gel (first dimension) and electrophoresed
under reducing conditions. Cleavage of the azo linkage in the Denny-
Jaffe cross-linker molecules was accomplished with three 15-min
soakings of the first dimension tube gel in fresh 0.2 mM sodium
dithionite solution. The gel tube was then equilibrated with 50 mM
Tris/HCl, pH 6.8, and 0.1% SDS prior to second dimension electro-
phoresis in a 7% SDS-PAGE slab gel and autoradiography.

Purification of #.1#-IL-6 by Gel Filtration High Performance Liquid Chromatography (HPLC)—Five hundred nanograms of #.1#-IL-6 in
200 μl of 0.2 M sodium phosphate, pH 7.5, were chromatographed on
a 7.5 x 60-cm Spherogel TSK 3000SW column (Altex) at a flow rate of
0.5 ml/min. One-milliliter fractions were collected, radioactiv-
ity was measured in a gamma-counter, and samples were analyzed
by SDS-PAGE.

RESULTS

Previous studies have characterized only a single, 80-kDa,
IL-6-binding membrane protein, termed gp80 (13, 26). How-
ever, in several affinity cross-linking reports (12, 17, 18) and
in our own preliminary studies, multiple IL-6-containing
cross-linked receptor complexes have been found. In order to
characterize IL-6-binding membrane proteins, we performed
affinity cross-linking studies using #.1#-IL-6 and the homobifunc-
tional cross-linking reagent disuccinimidyl suberate (12 Å).
In our studies, #.1#-IL-6 was allowed to bind for 1 h at
4 °C, and unbound ligand was removed prior to cross-linking.
In several cell lines, including U266, H929, U937, and
in phystoheagglutinin-activated peripheral blood lymphocytes,
this procedure consistently revealed a pattern of three major
#.1#-IL-6-containing receptor complexes with molecular masses
corresponding to 100, 120, and 150 kDa (Fig. 1). Identical results
were obtained using commercially prepared #.1#-IL-6 (data not shown).

More detailed studies were performed with the myeloma
cell lines U266 and H929, both of which express a large
number of IL-6 receptors (approximately 20,000/cell) (13, 16,
27). The 100-, 120-, and 150-kDa cross-linked complexes were
obtained with either reducing or nonreducing conditions, indi-
cating that interchain disulfide linkages were not responsi-

FIG. 1. Affinity cross-linking of #.1#-IL-6 to normal and
transformed human cells. 1 x 10^6 cells were incubated with 5 nM
#.1#-IL-6 at 4 °C for 60 min. After removal of unbound ligand,
the cells were cross-linked with 300 μg/ml disuccinimidyl suberate
for 15 min at 4 °C and analyzed by reducing SDS-PAGE and autoradi-
ography as described under "Materials and Methods." Autoradiogra-
phies were exposed for different periods to achieve similar intensities:
U266 and H929 lanes were exposed overnight, U937 for 3 days and
peripheral blood lymphocytes for 5 days.
able for the multiband pattern (Fig. 2A). Immunoprecipitation with affinity-purified, anti-IL-6 antibodies also produced the same three labeled bands (Fig. 2B). The specificity of the binding was confirmed by competition with unlabeled IL-6 during the binding step. Fig. 2C, lane 2, shows that a 200-fold excess of unlabeled IL-6 completely inhibited the binding of 125I-IL-6 to its receptor. The same affinity cross-linking pattern was also obtained using other homobifunctional cross-linkers including disuccinimidyl tartrate (6 Å) and dithiobis(succinimidyl propionate) (12 Å) (data not shown). An identical pattern was obtained when binding proceeded for 1 h at 37 °C (not shown), indicating the same associations were formed both at 4 and 37 °C. In addition, the 37 °C bands were less intense suggesting that binding at this temperature results in down-regulation of all three complexes.

Allowing for the 20-kDa mass of the IL-6 molecule, the formation of the three complexes suggested that as many as three IL-6-binding proteins of different molecular masses existed (80, 100, and 130 kDa). We assessed the possibility that the three bands represented differently glycosylated forms of a single IL-6 receptor molecule cross-linked to 125I-IL-6. Fig. 3 shows the result of deglycosylation and cross-linking experiments using a combination of 1) inhibition of N-linked glycosylation and 2) digestion of sialic acid and O-linked residues with glycosidases. Reductions in molecular weight occurred in all three cross-linked complexes. In addition, the continued presence of three distinct bands indicated that the multiple band pattern could not be explained by different levels of glycosylation on a single binding protein.

In order to further define the composition of the three complexes we employed an iodinated, heterobifunctional, cleavable cross-linker termed the 125I-Denny-Jaffe reagent (28-30). With this procedure, IL-6 is first covalently labeled with the 125I-Denny-Jaffe cross-linking reagent. After receptor interaction, the bound 125I-Denny-Jaffe-IL-6 is photocovalently linked to an adjacent protein. Upon cleavage of the cross-linker, the IL-6 is released and the 125I remains covalently attached to the IL-6-binding protein. Due to the sequential order of this procedure, cross-linking occurs only between the labeled IL-6 molecule and a single associated protein and cannot occur between adjacent unlabeled proteins. When analyzed by SDS-PAGE, the photocross-linked U266 cells yielded the three radioactive complexes (100, 120, and 150 kDa) that are seen with conventional cross-linking (Fig 4A). Cross-linked samples were then analyzed by two-dimensional SDS-PAGE in which the Denny-Jaffe cross-linker was cleaved prior to electrophoresis in the second dimension, thus resulting in the release of the IL-6 molecule and the transfer of 125I to the cross-linked protein. Fig. 4B shows that, after cleavage, two 80-kDa proteins (a and b) were released from positions on the diagonal that corresponded to the original 100- and 120-kDa complexes, whereas a separate 130-kDa protein (c) was released from the original 150-kDa complex. Several conclusions can be drawn from these results. Both the 100- and 120-kDa complexes include an 80-kDa membrane protein. Furthermore, since no additional proteins other than IL-6 can be cross-linked into the complex, this finding also demonstrates that the 120-kDa complex consists of an 80-kDa membrane protein in association with two 20-kDa molecules of IL-6. Equally important, these results also demonstrate that IL-6 associates directly with a 130-kDa membrane protein.

The association of the 80-kDa molecule with two IL-6 molecules to form the 120-kDa cross-linked complex was unexpected. We investigated the possibility that the 120-kDa complex was formed by the association of an 80-kDa receptor molecule with preexisting dimers of IL-6. SDS-PAGE and autoradiography of 125I-IL-6 yielded of only a single 20-kDa form, thus excluding the presence of covalent IL-6 dimers. To test for noncovalent dimers, we performed the photocross-linking procedure, with 125I-Denny-Jaffe-labeled IL-6, in the absence of cells. Photocross-linking of noncovalent dimers of IL-6 would be expected to generate 40-kDa cross-linked complexes. Fig. 5 shows that only the 20-kDa monomeric form of IL-6 was generated using the Denny-Jaffe procedure, thus indicating that IL-6 dimers did not exist prior to interaction with the 80-kDa membrane protein. This conclusion is also
complexes using IOD-l-Denny-Jaffe-derivatized IL-6. 4 × 10^6 U266 cells were incubated for 60 min in the dark at 4 °C with 5 nM of IL-6 which had been labeled with IOD-l-Denny-Jaffe cross-linker. After removal of the unbound ligand, cross-linking was completed by exposure to UV light as described under "Materials and Methods." Lane A, one half of the cell lysate was analyzed in one dimension on a 7% SDS-PAGE slab gel under reducing conditions. Lane B, the remaining lysate was electrophoresed in a 7% SDS-PAGE tube gel under reducing conditions. After electrophoresis the Denny-Jaffe cross-linker was cleaved as described under "Materials and Methods," and the gel was subjected to second dimension electrophoresis in a 7% SDS-PAGE slab gel and autoradiography.

**DISCUSSION**

Although only a single IL-6-binding membrane protein, gp80, has thus far been documented (15), several affinity cross-linking studies have reported the formation of multiple cross-linked complexes within the IL-6 receptor system. Cross-linked bands of 110, 160, and 190 kDa have been reported on the human lymphoblastoid line CESS (12), whereas 100-, 120-, and 200-kDa cross-linked complexes have been obtained using the human hepatoma, HepG2, and 3T3 fibroblasts transfected with the gp80 cDNA (17, 18). Although the gp80 IL-6 receptor was present in the latter studies, the exact composition of the individual cross-linked complexes has remained unknown. In this paper, we have structurally characterized the IL-6 receptor system on human cells using IOD-l-IL-6 and affinity cross-linking techniques. Our studies revealed the formation of three IL-6-membrane protein cross-linked complexes, on both normal and transformed cells, with apparent molecular masses of 100, 120, and 150 kDa, respectively. In initial studies, we determined that the three-band pattern was not the product of interchain disulfide linkages within the complexes. We also established that differential glycosylation of proteins within the cross-linked complexes was not responsible for the three-band pattern. We achieved identical results using homobifunctional crosslinkers ranging in length from 6 to 12 Å. Considering that the distance between interacting hormone/receptor residues at the binding interface of a related receptor is about 3 Å (31), the ability to bridge molecules with a 6-Å cross-linker suggests that the associated proteins were in direct contact with the IL-6 molecule. These results indicated that the association of IL-6 with its receptor may be more complex than previously described and prompted us to characterize the complexes in more detail.

To identify the membrane components that were cross-linked to IL-6 in each of the three complexes, we employed an iodinated, photoactivatable, and cleavable cross-linker, the IOD-l-Denny-Jaffe reagent, which, when coupled to IL-6, allows the transfer of IOD-l from the ligand to adjacent receptor components. Our analysis revealed several previously undocumented aspects of the IL-6 receptor complex. First, the 150-kDa complex includes IL-6 cross-linked to a 130-kDa membrane glycoprotein. Equally important, our data reveal that both the 100- and 120-kDa complexes include IL-6 cross-linked to an 80-kDa membrane protein. Since all cross-linking events with the Denny-Jaffe reagent must occur between the ligand (IL-6) and only one other protein, this result also demonstrates that the 120-kDa complex contains two IL-6 molecules cross-linked to a single 80-kDa glycoprotein.

In this study we found that IL-6 associates with two membrane glycoproteins whose molecular weights correspond to those of two transmembrane glycoproteins, gp80 and gp130, that have previously been shown to function in the IL-6 receptor system. The cDNAs for both gp80 and gp130 have been cloned, sequenced, and expressed in eukaryotic cells, and the mRNAs for both genes are expressed by the U266 myeloma cell line (14, 15). Forms of gp80 have been shown to directly bind IL-6 and to associate with cell surface gp130 in the presence of IL-6 (16). In addition, when expressed in IL-3-dependent cells, recombinant gp130 can interact with IL-6 plus soluble forms of recombinant gp80 to generate a growth signal thus implicating gp130 as a signal transducer (14). Although gp130 has been shown to participate in the generation of high affinity binding sites (14), cells expressing only recombinant gp130 fail to bind IL-6 (14). This observation has led to the hypothesis that gp130 does not bind or interact with IL-6 directly but instead associates with gp80 to stabilize...
the binding of IL-6 by gp80, thus converting gp80 a high affinity receptor (14, 16). The above hypothesis has also been supported by the absence, in previous structural studies, of any data showing a direct association of gp130 with IL-6. Assuming the 130-kDa glycoprotein described in our study is in fact gp130, the finding presented here suggests another equally plausible hypothesis: specifically, that gp130 does bind IL-6, presumably together with gp80 in the high affinity complex. The concept that gp130 can act as a ligand-binding molecule is supported by recent studies demonstrating that recombinant-expressed gp130 alone directly binds oncostatin M with low affinity (32).

The formation of 100- and 120-kDa cross-linked complexes has also been observed by Rose-John et al. (17, 18) who suggested that the 120-kDa complex may consist of gp80 plus two molecules of IL-6. In our experiments we have clearly demonstrated that the 100- and 120-kDa cross-linked complexes described here in fact contain one and two molecules of IL-6, respectively. We have also demonstrated that preexisting IL-6 dimers did not contribute to the formation of the 120-kDa complex. This suggests that the formation of the 120-kDa complex is a consequence of independent binding of the IL-6 molecules. Several models can be developed to explain the formation of the 100- and 120-kDa complexes. In one interpretation, the 120-kDa complex could be generated if gp80 possessed two binding sites for IL-6. We prefer an alternative model, also suggested by Rose-John et al. (17), in which two molecules of IL-6 would associate with two gp80 molecules to form a large tetrameric complex. Multiple cross-linking events within either of the possible models would generate both the 100- and 120-kDa cross-linked complexes. The last model is supported by affinity cross-linking studies of Rose-John et al. (17) in which a 200-kDa IL-6-containing cross-linked complex was generated in 3T3 fibroblasts transfected with gp80 cDNA. Our experiments do not allow us to distinguish between these possibilities. Whatever form the IL-6/gp80 association takes, it is possible that structures consisting of two molecules of IL-6 and one or two molecules of gp80 could exist independently or may interact with gp130 to engage the signal transduction pathway. Additional studies are needed to identify the mechanisms involved in this process.

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