Affinity Labeling of the Sialoglycopeptide Antimitogen Receptor*

(Received for publication, January 16, 1987)

Behrooz G. Sharifi and Terry C. Johnson†
From the Section of Oncology and Virology, Division of Biology, Kansas State University, Manhattan, Kansas 66506

An 18-kDa 125I-sialoglycopeptide growth inhibitor was covalently cross-linked to its binding site on intact cultured Swiss 3T3 cells by three bifunctional cross-linkers with short (dimethyl adipiminate), medium (disuccinimidyl suberate), and long (bis(2-succinimidoxy carbonyloxyethyl)sulfone) chain lengths. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography demonstrated a band of Mr ~168,000 regardless of which cross-linker was used. The labeling of this band was specific in that it was prevented by excess unlabeled inhibitor and the apparent molecular weight of the cross-linked receptor-ligand complex was unchanged by treatment with reducing agents. The efficiency of the cross-linking was increased by increasing pH, and the extent of covalent cross-linking was dependent on the concentration of the bifunctional reagent. Octyl glucoside and sodium dodecyl sulfate were effective in solubilizing the receptor while Triton X-100 did not extract the receptor from the plasma membrane. These observations suggest that the 168-kDa binding species represents the 125I-sialoglycopeptide cross-linked to a specific plasma membrane receptor and that the receptor does not appear to contain interchain disulfide bonds.

The initial biochemical step by which extracellular signals such as growth inhibitors or growth factors affect cellular proliferation and/or metabolism is the formation of specific ligand-receptor complexes on the plasma membrane of target cell populations. Understanding the biochemical basis of this important interaction requires a system in which the molecular properties of the two reactants, i.e., factor and receptor, are known. Although considerable progress has been made in understanding the biochemical and cellular consequences of the growth factor-receptor interactions, there is little information available on the interaction of growth inhibitors with the target cells.

We have isolated and purified to homogeneity a polypeptide from bovine brain that reversibly inhibits DNA and protein synthesis in various cell lines (1–3). The inhibitor is an 18-kDa glycopeptide which contains sialic acid. The purified glycopeptide is an acidic protein with a pI of 3.8 and has only one polypeptide chain with the biological activity residing on the polypeptide backbone (2).

We initially investigated the molecular basis for the sialo-

glycopeptide action by examining the binding of the inhibitor to the target cells (4). Binding studies revealed that 125I-sialoglycopeptide bound to a single class of saturable high affinity receptors specific for this ligand. Like the receptors for growth factors, receptors for the sialoglycopeptide inhibitor were found in relatively low numbers (3 × 10⁴/cell) on target cells (4). Binding of the inhibitor to the target cells was reversible, and most of the bound inhibitor dissociated from cell surface receptors as an intact molecule (4). In addition, we demonstrated that internalization of the inhibitor was not required in that a cell surface interaction was sufficient for the inhibitory activity of the sialoglycopeptide (5). Furthermore, we have shown that pretreatment of sensitive cells with the calcium ionophore A23187 renders the cells resistant to the inhibitory activity of the sialoglycopeptide suggesting that the inhibitor influences intracellular Ca²⁺ levels (6).

Covalent cross-linking of radiolabeled growth factors to their binding sites has been used to investigate the size and subunit structures of receptors for epidermal growth factor (7, 8), platelet-derived growth factor (9, 10), insulin (11), insulin-like growth factors I and II (12–14), α-TGF (15, 16), β-TGF (17), and other polypeptide hormones (18–20).

Although identification of several other putative inhibitors of cell division has been reported (21–24), there is little information on whether binding of these growth regulators involves specific interactions at the target cell surface or the nature of the receptors that might play a role in cell-cell communication. To understand the mode of action of the sialoglycopeptide growth inhibitor, it is important to gain information on the structural properties of the sialoglycopeptide receptor. In this study we have used three bifunctional cross-linkers to affinity label 125I-sialoglycopeptide binding sites on intact cultured Swiss 3T3 cells. We present evidence that the receptor for the sialoglycopeptide inhibitor has a molecular weight of ~150,000 and apparently lacks disulfide bonds to other subunits comprising, or being associated, with the sialoglycopeptide receptor.

MATERIALS AND METHODS

The bifunctional cross-linking reagents dimethyl adipiminate (DMA), disuccinimidyl suberate (DSS) and bis(2-succinimidoxy carbonyloxyethyl)sulfone (BSOCOES) were purchased from Pierce Chemical Co., and other chemicals were of the highest grade available. Swiss 3T3 cell lines were grown as monolayers at 37°C in a 95% air:5% CO₂ atmosphere, with Dulbecco’s modified Eagle medium supplemented with 10% calf serum. As previously described (2), the sialoglycopeptide inhibitor was isolated and purified to homogeneity from bovine cerebral cortex cells as described previously (1, 4). The major purification steps included chloroform/methanol extraction, DEAE ion-exchange chromatography, wheat germ lectin

* This study was supported by Grant CA27648 from the National Cancer Institute and by Grant CD-323 from the American Cancer Society. This is Contribution 67-252-J from the Kansas Agricultural Experiment Station, Kansas State University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

1The abbreviations used are: α-TGF, transforming growth factor; β-TGF, transforming growth factor; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMA, dimethyl adipiminate; DSS, disuccinimidyl suberate; BSOCOES, bis(2-succinimidoxy carbonyloxyethyl)sulfone.

15752
affinity chromatography, HPLC gel filtration, and hydroxylapatite
HPLC chromatography. Radioiodination of the sialoglycopeptide was
performed by a modification of the chloramine-T method, where the
specific radioactivity was $1 \times 10^6$ cpm/ng and where the inhibitory
activity is retained, as described previously (2).

For cross-linking studies, 3T3 cells were washed three times with a
balanced salt solution (pH 7.3) (4). The inhibitor-receptor binding
step was carried out in a final volume of 0.2 ml in conical polypro-
ylene centrifuge tubes (Fisher) each containing approximately 1 $\times$
10$^5$ cells and 4 nM $^{125}$I-sialoglycopeptide. Samples used to
determine nonspecific binding also contained 0.4 $\mu$M unlabeled
inhibitor. The samples were incubated for 1 h at 22°C with constant
mixing, and the cell-bound radioactivity was then separated from
radioactivity by centrifuging the cells through an oil mixture that
consisted of 84 parts silicone fluid and 16 parts light mineral oil (25).

To separate the receptor bound from the free $^{125}$I-sialoglycopeptide,
200 $\mu$l of the cell suspension were layered on top of the oil mixture
and the cells were centrifuged for 1 min at 700 X g. The supernatant
fluid was removed, and then the cells were resuspended in a balanced
salt solution (pH 7.3) or carbonate-saline buffer (pH 8.3) containing
one of the cross-linkers. The cross-linkers had been dissolved in
dimethyl sulfoxide and added to the cell suspension to a final concen-
tration of 0.06-1.0 $\mu$M. Reaction was allowed to occur for 30 min at
22°C with occasional mixing. Cross-linking reactions were termi-
nated by the addition of Tris to a final concentration of 50 $\mu$M to
counteract with only a faint high molecular weight band observed. We
reasoned that since the cross-linker reacts with the unproton-
able band was seen at an approximate molecular weight of
168,000 under both reducing (lane 1) and nonreducing (lane 2)
conditions. The intensity of the high molecular weight band was increased when the cross-linking was performed at

\[
\text{pH 8.3 (Fig. 1, panel A), compared to pH 7.3 (Fig. 1, panel B). To measure the effect of pH on the efficiency of cross-linking, the 168-kDa band was excised from the gel, and the radioactivity associated with this band was directly measured in a $\gamma$ counter. We have observed that the cross-linking at the higher pH (8.3) produced 5-fold more efficient radiolabel-
ing than cross-linking at the lower pH (7.3). Under these conditions 37% of the sialoglycopeptide bound to its receptor was cross-linked. Consequently, in subsequent experiments cross-linking was carried out at pH 8.3. A high concentration (0.4 $\mu$M) of unlabeled sialoglycopeptide (Fig. 1, lanes 3 and 6), incubated with the $^{125}$I-sialoglycopeptide during binding, redu-
ced the radiolabeling of the high molecular weight band by about 80% as determined by exciting the corresponding gel segments and counting their radioactivity with a $\gamma$ counter. Unreacted $^{125}$I-sialoglycopeptide was present in a band cor-
responding to 18 kDa, and free $^{125}$I migrated to the bottom of the gels. No other high molecular weight radioactive bands were detected in this gel.

The effects of various concentrations of DSS over a 16-fold concentration range (0.06-1.0 mM) were assayed for their ability to cross-link the $^{125}$I-sialoglycopeptide to intact cells. Only one radioactive band corresponding to $M_1 = 168,000$ was observed, and the intensity of this band was enhanced by increasing concentrations of DSS (data not shown). Conse-
quentially, we have used DSS at 1.0 mM concentration in the subsequent experiments. No other higher molecular weight radioactive bands were observed indicating that there is no detectable nonspecific cross-linking within this range of con-
centrations.

To investigate whether the length of the cross-linker has any measurable effect on the cross-linking of the inhibitor to the binding sites on intact cells, we also examined the cross-linking of the $^{125}$I-sialoglycopeptide to 3T3 cells using DMA and BSOCOES cross-linkers. DMA has a shorter (8.6 A)
chain length than DSS (11 Å), and BSOCOES has a longer (13 Å) chain length. The 168,000 molecular weight band was apparent in samples treated with both DMA and BSOCOES (Fig. 2). DMA (Fig. 2, panel A) was not as effective as DSS.
presence 2 or BSOCOES then treated with the cross-linkers as described under "Materials and Methods." Cross-linking was performed either with DMA (panel A) or BSOCOES (panel B). After cross-linking, the cell lysate was electrophoresed under reducing (lanes 1 and 4) or nonreducing (lanes 2 and 3) conditions.

The effect of various detergents on the extraction of the sialoglycopeptide receptor was investigated by solubilizing the cross-linked inhibitor with 0.5% (v/v) Triton X-100 and 0.5% (v/v) octyl glucoside, and the results were compared to the cells solubilized by 0.5% SDS. As demonstrated in Fig. 3, when cells were lysed with Triton X-100 and then the supernatant fluid was subjected to electrophoresis the 168-kDa band had a very low intensity (Fig. 3, lane 2). The Triton X-100 pellet, however, contained the majority of the radioactive material (Fig. 3, lane 3). Octyl glucoside, on the other hand, was as effective as SDS in solubilizing the sialoglycopeptide receptor from 3T3 cell surface membrane (Fig. 3, lane 4).

**DISCUSSION**

We have previously reported that the binding of the 125I-sialoglycopeptide inhibitor to 3T3 cells was specific and that the inhibitor bound reversibly to a high affinity receptor (4). However, the exact nature of the interaction between the inhibitor and the component(s) responsible for conveying the inhibitory signal to the target cells remained unclear. In the present paper, we report that structural identification of a sialoglycopeptide receptor from 3T3 cell surface membrane (Fig. 3, lane 4).

FIG. 2. Affinity labeling of the sialoglycopeptide receptors with DMA and BSOCOES cross-linkers. Cells were incubated with 125I-sialoglycopeptide in the absence (lanes 1, 2, 4 and 5) or presence (lanes 3 and 6) of an excess unlabeled inhibitor and were then treated with the cross-linkers as described under "Materials and Methods." Cross-linking was performed either with DMA (panel A) or BSOCOES (panel B). After cross-linking, the cell lysate was electrophoresed under reducing (lanes 1 and 4) or nonreducing (lanes 2 and 3) conditions. (Fig. 1) although BSOCOES (Fig. 2, panel B) was as effective as DSS in the formation of the 168-kDa band without increasing nonspecific cross-linking. In both cases the presence of excess unlabeled inhibitor (Fig. 2, lanes 3 and 6) prevented the radiolabeling of the 168-kDa band. The apparent molecular weight of the radioactive band did not vary with the different cross-linkers, and we could not detect any other high molecular radioactive band associated with the sialoglycopeptide inhibitor when these cross-linkers were used.

The effect of various detergents on the extraction of the sialoglycopeptide receptor was investigated by solubilizing the cross-linked inhibitor with 0.5% (v/v) Triton X-100 and 0.5% (v/v) octyl glucoside, and the results were compared to the cells solubilized by 0.5% SDS. As demonstrated in Fig. 3, when cells were lysed with Triton X-100 and then the supernatant fluid was subjected to electrophoresis the 168-kDa band had a very low intensity (Fig. 3, lane 2). The Triton X-100 pellet, however, contained the majority of the radioactive material (Fig. 3, lane 3). Octyl glucoside, on the other hand, was as effective as SDS in solubilizing the sialoglycopeptide receptor from 3T3 cell surface membrane (Fig. 3, lane 4).

FIG. 3. Effect of various detergents on the solubilization of the inhibitor receptor. Binding of the sialoglycopeptide to 3T3 cells was performed as described. After binding, 3T3 cells were lysed with a 0.05 mM solution of Tris buffer containing 0.5% SDS (lane 1); supernatant fluid, 0.5% Triton X-100 (lane 2); pellet, 0.5% Triton X-100 (lane 3); and 0.5% octyl glucoside (lane 4). The lysates were analyzed by SDS-PAGE as described above.

The effect of various detergents on the extraction of the sialoglycopeptide receptor was investigated by solubilizing the cross-linked inhibitor with 0.5% (v/v) Triton X-100 and 0.5% (v/v) octyl glucoside, and the results were compared to the cells solubilized by 0.5% SDS. As demonstrated in Fig. 3, when cells were lysed with Triton X-100 and then the supernatant fluid was subjected to electrophoresis the 168-kDa band had a very low intensity (Fig. 3, lane 2). The Triton X-100 pellet, however, contained the majority of the radioactive material (Fig. 3, lane 3). Octyl glucoside, on the other hand, was as effective as SDS in solubilizing the sialoglycopeptide receptor from 3T3 cell surface membrane (Fig. 3, lane 4).

The effect of various detergents on the extraction of the sialoglycopeptide receptor was investigated by solubilizing the cross-linked inhibitor with 0.5% (v/v) Triton X-100 and 0.5% (v/v) octyl glucoside, and the results were compared to the cells solubilized by 0.5% SDS. As demonstrated in Fig. 3, when cells were lysed with Triton X-100 and then the supernatant fluid was subjected to electrophoresis the 168-kDa band had a very low intensity (Fig. 3, lane 2). The Triton X-100 pellet, however, contained the majority of the radioactive material (Fig. 3, lane 3). Octyl glucoside, on the other hand, was as effective as SDS in solubilizing the sialoglycopeptide receptor from 3T3 cell surface membrane (Fig. 3, lane 4).

**DISCUSSION**

We have previously reported that the binding of the 125I-sialoglycopeptide inhibitor to 3T3 cells was specific and that the inhibitor bound reversibly to a high affinity receptor (4). However, the exact nature of the interaction between the inhibitor and the component(s) responsible for conveying the inhibitory signal to the target cells remained unclear. In the present paper, we report that structural identification of a major receptor component of ~150,000 molecular weight on the surface of a sensitive 3T3 cell that specifically binds the sialoglycopeptide. SDS-polyacrylamide gel electrophoresis, under reducing and nonreducing conditions, illustrated the apparent molecular weight of the cross-linked complex to be approximately 168,000, yielding a receptor size of approximately 150,000 by subtraction of the molecular weight of the sialoglycopeptide. This calculation was based on the assumption that the cross-linking occurred between one molecule of inhibitor per molecule of receptor. Although the inhibitor cross-linked to the receptor at pH 7.3, increasing the pH to 8.3 resulted in a marked increase in the radiolabeling efficiency (Fig. 1). These results suggested that the amino groups of the sialoglycopeptide receptor and/or inhibitor were stabilized in the protonated form by interacting with the glutamic or aspartic acid residues. These types of interactions could increase the pK of the amino groups of the receptor and/or inhibitor that requires a higher pH to deprotonate amino groups.

Results of experiments using various concentrations of cross-linking reagents demonstrated the lack of nonspecific or random cross-linking of the inhibitor with other membrane components. In addition, the 125I-sialoglycopeptide was cross-linked to the binding site on intact 3T3 cells by two additional bifunctional cross-linkers with shorter (DMA) or longer (BSOCOES) chain length than DSS (Fig. 2). In all cases a single radioactive high molecular weight band was observed at 168,000. This apparent size was unchanged by a disulfide reducing agent 2-mercaptoethanol, suggesting that the receptor might be composed of a single chain polypeptide. We cannot rule out, however, the possibility that the receptor was composed of subunits and these subunits were covalently bound together during the cross-linking reaction. The results with various cross-linkers also suggest that the association between the inhibitor and the receptor was specific since control experiments with excess unlabeled sialoglycopeptide did not result in a radiolabeled high molecular weight band (Fig. 2, lanes 3 and 6). Results of the experiment using various detergents to solubilize the sialoglycopeptide receptor (Fig. 3) demonstrated that Triton X-100 was not capable of extracting the sialoglycopeptide receptor (Fig. 3, lane 2); however, octyl glucoside and SDS were effective in extracting the sialoglycopeptide receptor from the plasma membrane (Fig. 3, lanes 1 and 4). It has been demonstrated that the EGF receptor is predominantly associated with the Triton X-100-insoluble cytoskeleton fraction in human A431 carcinoma cells (26). It is possible that the sialoglycopeptide receptor also might be associated with the cytoskeleton. The results of the detergent
experiment also can be used as a guide to select an appropriate detergent to solubilize the sialoglycopeptide receptor for further chemical/physical characterization.

Does the 150-kDa binding protein mediate the inhibitory activity of the sialoglycopeptide? We have previously demonstrated that the dose response and kinetics of binding of the sialoglycopeptide to this specific receptor were directly correlated with the biological activity. In addition, we have shown that the kinetics of dissociation of the bound inhibitor closely follows the kinetics of cell recovery (4). Thus, the identification of only one binding component on the surface of sensitive 3T3 cells suggests that this receptor is involved in transmitting the inhibitory signal to the target cells. This conclusion is particularly important in light of recent findings about the thrombin receptor (27). It has been shown that biologically active thrombin binds, with high affinity, to a specific cell surface thrombin, and affinity labeling revealed the specific labeling of a major thrombin-binding site of 150 kDa. Affinity and maximal binding capacity of inactive thrombin to its receptor, however, paralleled that of active thrombin, and both active and inactive thrombin affinity-labeled receptors had similar molecular weights (27). The binding of inactive thrombin did not, however, interfere with the mitogenic activity of the active thrombin. Thus, it was suggested that the identified thrombin receptor did not mediate the induction of the cellular mitogenic response (27). The presence of a specific high affinity binding component on the cell surface, therefore, does not necessarily mean that it transmits the biological signal of the ligand. The finding that the receptor occupancy by the sialoglycopeptide growth inhibitor correlated with the inhibitory activity of the sialoglycopeptide (4) indicates that the affinity-labeled receptor is involved in conveying the inhibitory signal to the cells.

Cross-linking studies with various growth factors demonstrated that most of the growth factor receptors with the exception of the insulin, insulin-like growth factor I, and insulin-like growth factor II receptors are composed of a single polypeptide chain (28). There is, however, little information available on the identification of growth inhibitor receptors. As of yet, the only growth inhibitor receptor that has been identified is the β-TGF receptor. Using DSS as a cross-linker, it has been shown that the β-TGF receptor is a monomeric polypeptide with $M_r = 280,000$ (17). It is not clear, however, whether the 286,000 affinity-labeled receptor mediates the biological activity of β-TGF.

The studies reported here demonstrate that the sialoglycopeptide receptor consists of a 150-kDa binding component. It is obvious that other cell surface components may interact with the sialoglycopeptide receptor and may be dissociated upon solubilization and electrophoresis of affinity-labeled cells. Further characterization and isolation of the sialoglycopeptide receptor is needed to address this and other questions on the structure of the sialoglycopeptide receptor. The information derived from this study should help to initiate an effort to isolate and chemically characterize the sialoglycopeptide receptor.

REFERENCES