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Identification of Two Oligodeoxyribonucleotide Binding Proteins on Plasma Membranes of Human Cell Lines

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ABSTRACT. Two oligodeoxyribonucleotide (oligodN) binding proteins of approximately 100–110 kDa were identified in the plasma membranes of human HL-60, HepG2, H1, and KB cells by a photolabeling technique. Solubilization of cellular membranes with a nonionic detergent did not interfere with the binding of these two proteins to oligodNs, and both proteins were susceptible to serine protease action. The binding affinities of these two proteins to oligodNs were found to be similar; Scatchard plot analysis revealed the K_d for phosphodiester (PO) 21-mer oligodeoxycytidine to be 60 nM and binding sites numbered approximately 1.2×10^6/cell for HepG2 cells. Both phosphorothioate (PS) and PO oligodNs could bind to these two proteins with the binding affinity for PS oligodNs being much stronger than that for PO oligodNs. The binding to oligodNs was affected by the ionic strength of the reaction. Dextran sulfate, tRNA, and double-stranded DNA inhibited the binding of oligodNs, whereas ATP, ADP, AMP, and TTP had no effect. Given their high affinity for oligodNs, these membrane proteins may play an important role in the action of oligodNs. BIOCHEM PHARMACOL 51;4:431–436, 1996.

KEY WORDS. oligodeoxynucleotide binding proteins; antisense; oligodeoxynucleotide uptake

OligodNs‡ are considered as a new drug entity due to their potential as antisense, antigene, or antiprotein molecules [1]. The mechanism of cellular uptake of oligodNs can be affected by chemical modifications of the phosphodiester linkage within the molecule. The uptake of ionic molecules such as PO oligodNs and PS oligodNs occurs via both fluid-phase pinocytosis and adsorptive endocytosis [2–5]. The cellular plasma membrane proteins involved in the process of uptake and action of PO and PS oligodNs are not well characterized. More than 60 different types of proteins have been identified in human plasma membranes, and some of them bind nucleic acids and/or oligodNs. Bennett *et al.* [6] described a 30-kDa DNA binding protein on the surfaces of monocytes, B cells, and T cells by using biotinylated DNA probes. ^{32}P-Labeled calf thymus DNA was used by Gasparro *et al.* [7] to show that cell membranes of normal lymphocytes contain three high affinity DNA proteins of 28, 59 and 79 kDa. It was suggested that these proteins are involved in the natural maintenance of cell membrane DNA [7]. Loke *et al.* [3] employed oligo(dT)-cellulose to isolate an 80-kDa protein capable of binding oligodNs from plasma membrane of HL-60 cells labeled by iodine. Similarly, two proteins of 79 and 90 kDa were detected by Yakubov *et al.* [4] in mouse fibroblasts treated with derivatives of alkylating oligodNs. All of these proteins could play a role in the action of oligodNs. In the present study, we describe two proteins of approximately 100–110 kDa as major oligodN binding proteins on human cell plasma membranes and some of their binding characteristics.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade or better. RPMI 1640 medium and dialyzed fetal bovine serum were purchased from JRH Biosciences, Lenexa, KS. Kanamycin was obtained from the Sigma Chemical Co., St. Louis, MO.

Oligonucleotides

The 21-mer PS oligodN (SdN_{21}) with the base sequence 5′-GCC-GAG-GTC-CAT-GTC-GTA-CGC-3′, the 21-mer PO oligodeoxycytidine (dC_{21}), and the PS oligodeoxycytidine of 21, 28, and 35 bases long (SdC_{21}, SdC_{28}, and SdC_{35}) were synthesized and purified as described previously [8, 9].

Cells

The human cell lines used included the B lymphoma cell line H1, the epidermoid carcinoma cell line KB, the promyelocytic

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‡ *Abbreviations:* oligodN, oligodeoxyribonucleotide; PO oligodN, phosphodiester oligodeoxyribonucleotide; PS oligodN, phosphorothioate oligodeoxyribonucleotide; dC_{21}, 21-mer oligodeoxycytidine; SdC_{21}, 21-mer phosphorothioate oligodeoxycytidine; SdC_{28}, 28-mer phosphorothioate oligodeoxycytidine; SdC_{35}, 35-mer phosphorothioate oligodeoxycytidine; PMSF, phenylmethylsulfonyl fluoride; and DTT, dithiothreitol.

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leukemia cell line HL-60, and the hepatoma cell line HepG2. HepG2 cells were grown in Minimal Essential Medium supplemented with 10% fetal bovine serum. All other cell lines were cultured in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μg/mL kanamycin at 37° in a humidified atmosphere containing 5% CO_2.

Preparation of Plasma Membranes

Plasma membranes were extracted as previously described by Naito *et al.* [10]. Briefly, cells in the exponential phase of growth were washed with PBS and disrupted in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.5 μg/mL pepstatin A and 0.5 μg/mL leupeptin) by using a ground glass homogenizer in an ice bath. The homogenate was then centrifuged at 1000 *g* for 10 min. The supernatant was overlaid on a 35% sucrose solution and centrifuged for another 60 min at 100,000 *g*. The pellets were resuspended and stored at −70° until used. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

Photolabeling of Plasma Membranes

The oligodNs were 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase according to an established procedure [11]. The photolabeling of plasma membranes was performed as previously described [12]. Briefly, 5 μg of membrane proteins was mixed with 0.1 μM radiolabeled oligodN in 50 μL of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 2% glycerol, 0.02% NP-40, 0.2 mM PMSF) for 2 min at 22°. The membranes were then irradiated at 254 nm by using the Stratalinker UV Crosslinker 1800 for 1 min at 4°. Photolabeled membrane proteins were resolved on 10% SDS-PAGE [13].

Scatchard Plot Analysis

Binding experiments were performed by incubating different concentrations of ^{32}P-labeled dC_{21} with 2 μg of membrane proteins in a final volume of 50 μL of buffer A for 5 min at 22°. The membranes were then photolabeled as above. Free and bound oligodNs were separated by filtration on Whatman GF/C glass microfiber membranes that were washed with 15 mL of PBS and dried. Membrane-associated radioactivity was quantified by liquid scintillation spectrometry. Non-specific binding was evaluated by incubating membrane extracts in the presence of a 10-fold excess of unlabeled oligodNs. The data obtained were analyzed by a Scatchard plot [14] to determine the B_{max} and K_d values.

Inhibition of ^{35}S-Labeled SdC_{28} Uptake by Other OligodNs in HepG2 Cells

Inhibition of cellular uptake of [^{35}S]SdC_{28} (180 mCi/mmol) was measured using a previously described procedure [15]. Briefly, HepG2 cells were incubated with 0.5 μM [^{35}S]SdC_{28} for 1 hr at 37°, in the presence of a 2 μM concentration of unlabeled SdC_{21} or SdC_{35}, followed by removal of the medium and three washes with ice-cold Krebs–Ringer buffer, pH 7.5. The membrane-bound ligands were eluted with 0.2 M acetic acid and 0.5 M sodium chloride, pH 2.5, after 10 min at 4°. The intracellular radioactivity was measured by liquid scintillation spectrometry.

RESULTS

Identification of OligodN Binding Proteins on Human Cellular Plasma Membranes and Effect of Nucleotides and Nucleic Acids on the Binding Activity

^{32}P-labeled 21-mer PO oligodNs were incubated with plasma membranes prepared from HepG2 cells. After photolabeling, proteins were separated by SDS-PAGE. Two proteins whose size ranged from 100 to 110 kDa were detected as the major PO oligodN binding proteins (Fig. 1A). The binding to PO

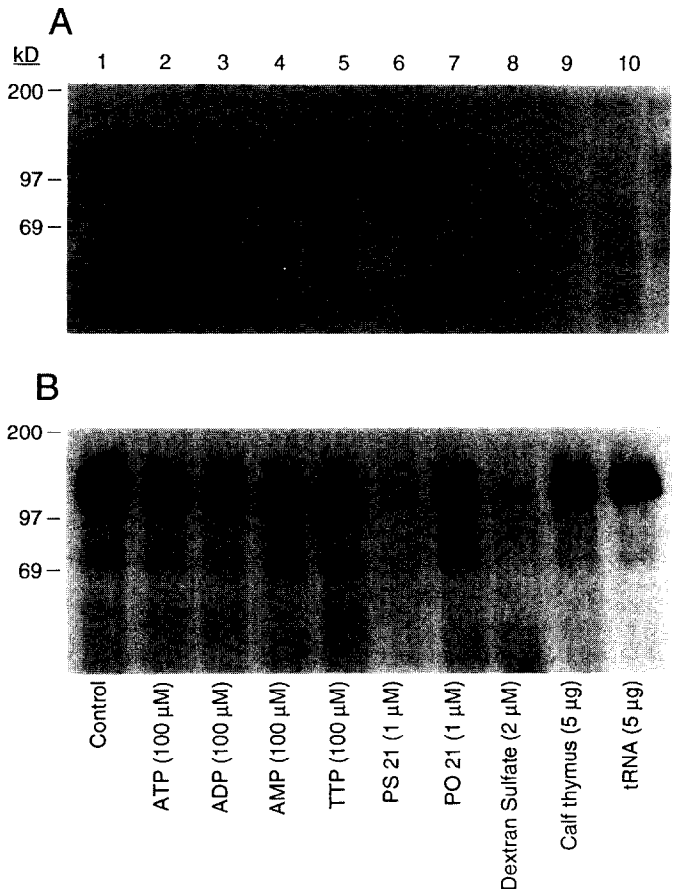

FIG. 1. Demonstration of oligodN binding proteins and the effects of different compounds on binding to oligodNs. Plasma membranes from HepG2 cells were photolabeled with ^{32}P-labeled oligodNs as described in Materials and Methods. Briefly, membrane proteins (5 μg) were incubated for 2 min with 0.1 μM ^{32}P-labeled 21-mer PO oligodNs (A) or ^{32}P-labeled 21-mer PS oligodNs (B) in the presence of ATP, ADP, AMP, TTP, PS 21-mer, PO 21-mer, dextran sulfate, calf thymus DNA, and tRNA at the concentrations indicated. The membranes were then irradiated for 1 min and analyzed on 10% SDS-PAGE. Molecular size markers are indicated (kDa).

oligodNs was not decreased in the presence of 1000-fold excess of ATP, ADP, AMP, and TTP, but was decreased by 21-mer PO oligodN or 21-mer PS oligodN, double-stranded DNA, tRNA, and dextran sulfate. The same results were observed with ^{32}P-labeled 21-mer PS oligodNs (Fig. 1B); however, the binding was not decreased by double-stranded DNA, tRNA, or 21-mer PO oligodNs. This suggests that the proteins have a stronger binding affinity for PS oligodNs than for PO oligodNs.

Degradation and Solubilization of the OligodN Binding Proteins

Both oligodN binding proteins were susceptible to serine protease action. The 100-110 kDa oligodN binding proteins could not be detected when cell plasma membranes were prepared in the absence of 0.5 mM PMSF, an inhibitor of serine protease (Fig. 2A). Instead, smaller oligodN binding proteins whose size ranged from 40 to 90 kDa appeared. Moreover, the presence of the protease inhibitors leupeptin and pepstatin was not required to protect the two proteins from degradation. Solubilization of plasma membranes with nonionic detergents NP-40 and/or *n*-octylglucoside did not alter the binding affinity of those two proteins to oligodNs (Fig. 2B).

Effects of Mg^{2+}, Ca^{2+} and Na^{+} on the Binding of OligodNs to the Plasma Membranes

The binding of the two proteins to oligodNs was investigated in the presence of increasing concentrations of Mg^{2+}, Ca^{2+}, and Na^{+}. The binding affinity of the proteins to oligodNs was poor when Mg^{2+}, Ca^{2+}, or Na^{+} was omitted from the reaction (Fig. 3). However, optimal binding was observed in the presence of either 0.5 mM $MgCl_2$ or 0.5 mM $CaCl_2$ (Fig. 3A) or in the presence of 100 mM NaCl (Fig. 3B).

Scatchard plot of dC_{21} Binding to Plasma Membrane Proteins Isolated from HepG2 Cells

HepG2 membrane proteins (2 μg) were incubated with various concentrations of ^{32}P-labeled 21-mer PO oligodeoxycyti-

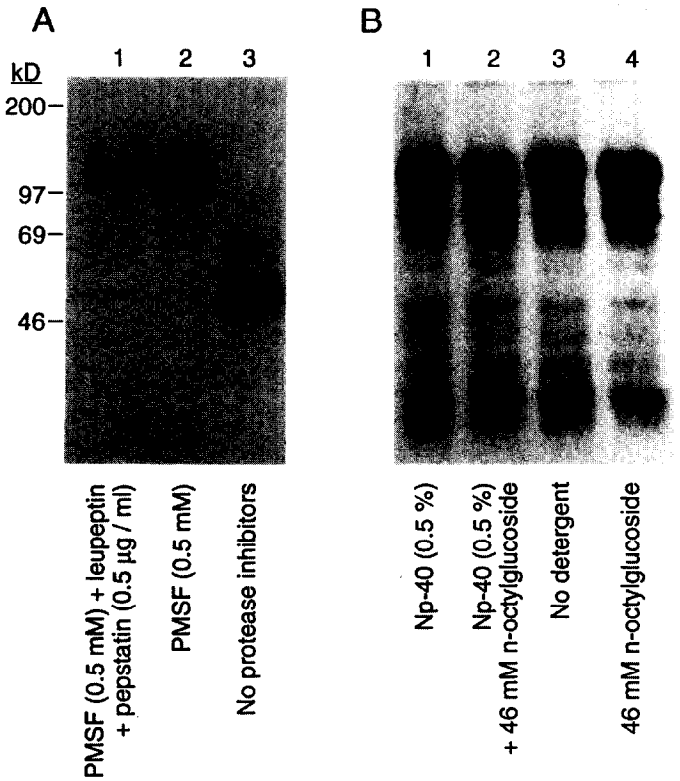

FIG. 2. Degradation and solubilization of the oligodN binding proteins. (A) 0.1 μM ^{32}P-labeled SdN_{21} was incubated with 5 μg of plasma membranes prepared in the presence or absence of different protease inhibitors. Lane 1: the membrane proteins were prepared with PMSF, pepstatin and leupeptin; lane 2: with PMSF alone; and lane 3: with no protease inhibitors. (B) plasma membranes (5 μg) were solubilized with different nonionic detergents and incubated with a 0.1 μM concentration of ^{32}P-labeled SdN_{21}. Lane 1: reaction in the presence of the nonionic detergent NP-40; lane 2: in the presence of NP-40 and *n*-octylglucoside; lane 3: no detergents (control); and lane 4: in the presence of *n*-octylglucoside.

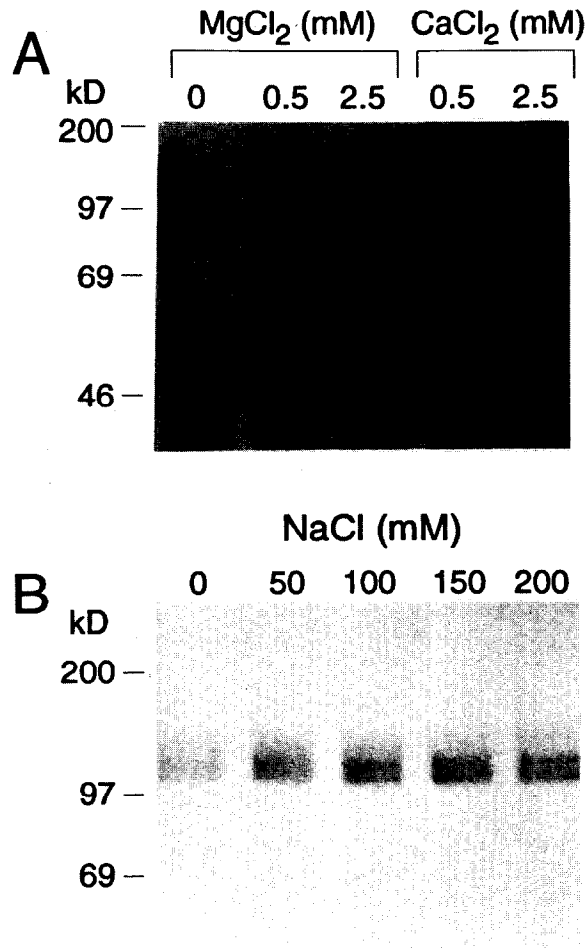

FIG. 3. Effects of Mg^{2+}, Ca^{2+}, and Na^{+} on the binding of oligodNs to plasma membranes. To determine the requirement of Na^{+}, Mg^{2+} and Ca^{2+} for binding, a 0.1 μM concentration of ^{32}P-labeled SdN_{21} was incubated in the presence of the indicated concentrations of $MgCl_2$, $CaCl_2$, and NaCl.

dine (dC_{21}). The results are presented in the form of a Scatchard plot as shown in Fig. 4. The calculated dissociation constant (K_d) was 60 ± 24.4 nM, and the maximal binding (B_{max}) was 77 ± 10 pmol/mg protein. When 21-mer PS oligodeoxycytidine (SdC_{21}) was used as a competitor of radiolabeled dC_{21}, the constant of inhibition (K_i) was found to be around 0.5 ± 0.14 nM. These data strongly indicate, as suggested previously, that PS oligodNs have a better binding affinity than PO oligodNs to the two proteins.

Identification of Different Amounts of OligodN Binding Proteins in Different Human Cell Surfaces and Their Correlation in Uptake of OligodNs

To explore if these two proteins exist on the surface of cells, intact cells from human cell lines HepG2, KB, HL-60, and H1 were analyzed. The putative proteins were found in the plasma membranes of all of the cell lines (Fig. 5A); additionally, these proteins were not detectable when cells were pretreated by trypsin, suggesting that these binding proteins are not in the inner aspect of the plasma membrane (data not shown). The intensity of the autoradiographic bands was quantitated by a scanning densitometer, and a good correlation between the amount of uptake of oligodNs [5] and the amount of oligodN binding proteins in those cell lines is shown in Fig. 5B.

Inhibition of Binding and Uptake of SdC_{28} by Shorter and Longer OligodNs

Unlabeled PS oligodeoxycytidines, either 21 or 35 bases long (SdC_{21}, SdC_{35}), were used as competitors of the binding of

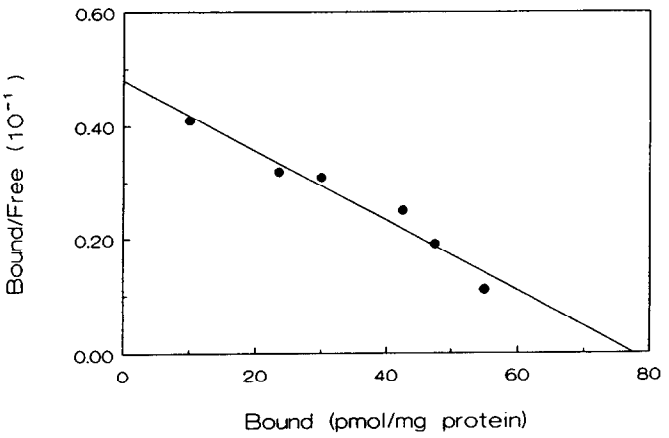

FIG. 4. Scatchard plot analysis of the interaction of dC_{21} and plasma membrane. B_{max} and K_d values were determined by the method of Scatchard as described in Materials and Methods. Briefly, 2 μg of plasma membrane was incubated with ^{32}P-labeled dC_{21} at 22° for 5 min. The samples were then irradiated, and the oligodNs bound to proteins were separated from free oligodNs by filtration on glass microfiber membranes. The membranes were washed with PBS, and membrane-associated radioactivity was measured by liquid scintillation spectrometry. Non-specific binding was evaluated by repeating the assay in the presence of a 10-fold excess of unlabeled oligodNs. The data were plotted, and the values for B_{max} and K_d were calculated. The experiment was repeated three times, and the result was reproducible.

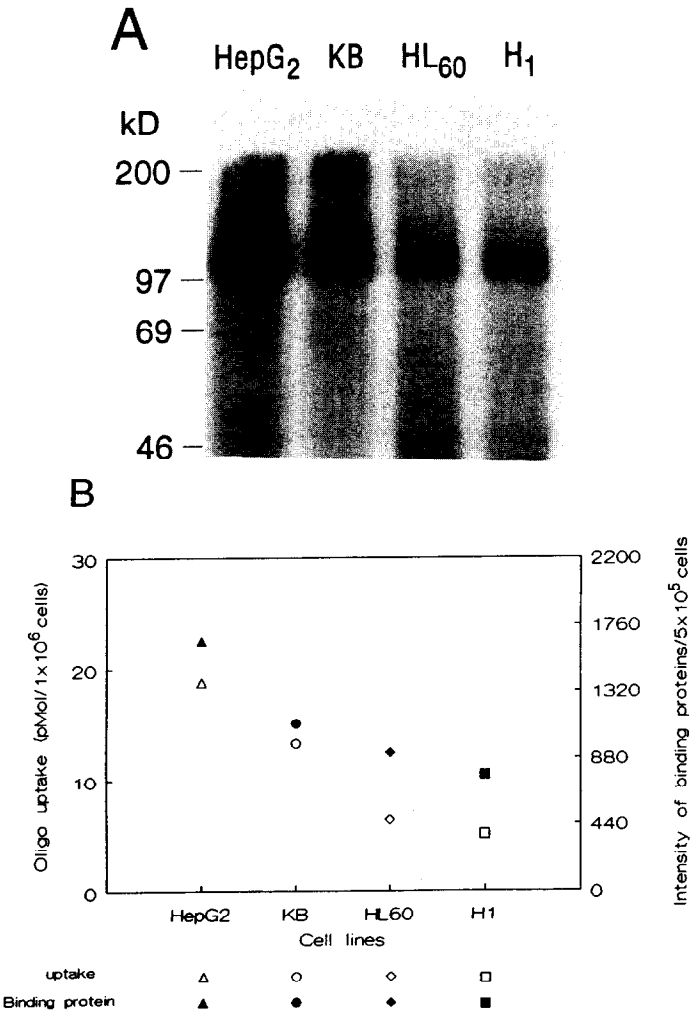

FIG. 5. Identification of the amount of oligodN binding proteins in different cell surfaces and correlation with the amount of uptake of oligodNs. Cells (5 × 10^5) from HepG2, KB, HL-60, and H1 cell lines were incubated with a 0.1 μM concentration of ^{32}P-labeled SdC_{28} for 2 min at 22°. The cells were irradiated for 1 min, lysed with 1% SDS, and analyzed by 10% SDS-PAGE. The identified proteins were able to be detected in all cell lines; however, the amounts of these proteins varied in the different cell lines (A). The intensity of the autoradiographic bands was quantitated by a scanning densitometer, and the correlation between the amount of uptake of oligodNs [5] and the amount of oligodN binding proteins in those cell lines is shown (B).

^{32}P-labeled SdC_{28} to HepG2 cells. As shown in Fig. 6, the longer oligodN (SdC_{35}) was able to inhibit the binding better than the shorter one (SdC_{21}). Similarly, the same oligodNs competed for uptake with ^{35}S-labeled SdC_{28} in HepG2 cells in a length-dependent manner.

DISCUSSION

Plasma membrane proteins that may be involved in oligodeoxyribonucleotide uptake are poorly understood. By using a photolabeling method, we were able to detect two oligodN binding proteins, approximately 100–110 kDa in size, in four different human cell lines. An 80-kDa protein isolated from

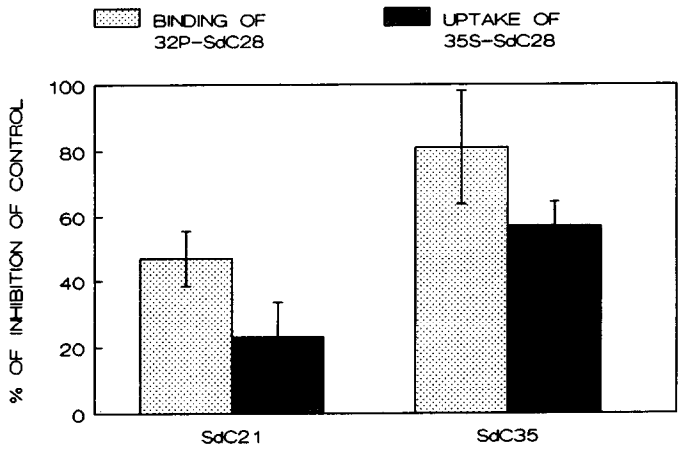

FIG. 6. Correlation between binding and uptake inhibition of SdC_{28} by shorter and longer oligodNs. The binding inhibition of ^{32}P-labeled SdC_{28} was performed by incubating HepG2 cells with a 0.1 μM concentration of ^{32}P-labeled SdC_{28} in the presence of a 0.4 μM concentration of unlabeled SdC_{21} or SdC_{35} for 5 min at 4°. The cells were then irradiated and analyzed by 10% SDS-PAGE. The intensity of the autoradiographic bands was quantitated by a scanning densitometer, and the rate of inhibition was reported as percent of the control. The uptake inhibition of ^{35}S-labeled SdC_{28} in HepG2 cells was performed by incubating the cells with a 0.5 μM concentration of ^{35}S-labeled SdC_{28} for 1 hr at 37° in the presence of 2 μM unlabeled SdC_{21} or SdC_{35}, followed by removal of the medium and three washes with ice-cold Krebs–Ringer buffer, pH 7.5. The membrane-bound ligands were eluted in a solution of 0.2 M acetic acid and 0.5 M sodium chloride, pH 2.5, after 10 min at 4°. After the removal of acetic acid, the intracellular radioactivity was measured by liquid scintillation spectrometry and interpreted as the intracellular ^{35}S-labeled SdC_{28}. Data represent the means ± SD from three experiments. The statistical evaluation of the difference of the inhibitory effect on SdC21 and SdC35 was significant ($P < 0.05$).

HL-60 cells by Loke *et al.* [3] was suggested to be an oligodN receptor. The role of this protein in the uptake of oligodNs is unclear; however, mononucleotide, as well as tRNA and plasmid DNA, blocks intracellular accumulation of acridine-labeled oligodNs in the HL-60 cells [3]. In contrast, our studies indicated that even at a very high concentration AMP, ADP, and ATP did not inhibit $[^{35}S]SdC_{28}$ uptake in HepG2 cells (data not shown). Furthermore, AMP, ADP, ATP, and TTP did not decrease the binding of either PO or PS oligodNs to the 100–110 kDa proteins. Calf thymus DNA and tRNA were each able to compete for the binding of PO oligodNs (Fig. 1). These results suggest that the proteins described in the present work not only differ from the 80-kDa protein based upon size, but also exhibit a different oligodN binding behavior. However, it should be noted that the oligodNs used by Loke *et al.* [3] were acridine labeled at the 5′ end, whereas the oligodNs used in our studies were PO and PS oligodNs. It is possible that 5′ end chemical modification of oligodNs by acridine, may change the species of proteins to which the oligodNs are able to bind. Moreover, in the absence of protease inhibitors, oligodN binding proteins with sizes ranging from 40 to 90 kDa could be observed (Fig. 2). These are most likely degraded forms of the 100–110 kDa proteins. Thus, it is possible that the 80 kDa protein previously described by others [3] is the degradation product of the 100–110 kDa protein described here.

An important question raised by the present study concerns the possible relationship between those two oligodN binding proteins on cellular plasma membrane, which exhibit similar characteristics of binding to oligodNs despite differences in their molecular weight. Scatchard plot analysis of oligodN binding to plasma membrane of HepG2 cells gave a linear line, suggesting that binding affinity of both proteins to oligodNs is similar (Fig. 4). Photolabeling experiments also supported this conclusion (data not shown). It is possible that these two proteins may be derived from a single precursor through different modifications. We leave this question open. Molecular characterization of these two proteins is required to address this question.

The two oligodN binding proteins were present on the plasma membrane of all the cells examined, although the quantity of the two proteins, based on their ability to bind oligodNs, differed among the cell lines (HepG2 > KB > HL60 > H1) (Fig. 5). It was demonstrated previously by Gao *et al.* [5] that the amount of uptake of oligodNs by cells is dependent on cell type. There was a good correlation between the amount of uptake of oligodNs and amount of oligodN binding proteins in those cell lines (Fig. 5B). Moreover, as shown in Fig. 6, the inhibition of binding of ^{32}P-labeled SdC_{28} by longer and shorter oligodNs correlated with the inhibition of the uptake of ^{35}S-labeled SdC_{28} by the same oligodNs. This suggests that the amount of oligodN binding proteins on the surface of cellular plasma membranes is related to the extent of oligodN uptake into the cells, which supports the idea that these proteins may be involved in oligodN uptake.

Fluid-phase pinocytosis and adsorptive endocytosis have been described as mechanisms of PO and PS oligodN uptake. At low oligodN concentration, adsorptive endocytosis is believed to play a major role in PO and PS oligodN uptake [3, 4], although other investigators have proposed that the majority of oligodN internalization proceeds via fluid-phase pinocytosis [15]. We previously reported that the uptake of SdC_{28} involves both mechanisms, since it is partially dependent on both temperature and energy [5]. Whether the two proteins identified are involved in the oligodN uptake and which process mediates the uptake of oligodNs are being investigated currently.

It was demonstrated by our study that the backbone modification of oligodeoxycytidine with sulfur could enhance the affinity of binding to those two proteins by at least 100-fold. Presently, we are also investigating the impact of the length and the base composition of the oligodNs. Some preliminary results indicate that these two properties can alter the capacity of binding of the oligodNs to the two membrane proteins. Although dC, SdC homooligonucleotides, and sequence specific dN, SdN oligos showed binding ability to these membrane proteins, the K_i values for SdC_{21} and SdN_{21} binding to the identified proteins were significantly different (unpublished data); this suggested that the binding affinity of oligodNs to these proteins may be altered by different sequences of oligodNs although the binding ability of the oligodNs to the proteins is not sequence dependent.

In summary, two oligodN binding proteins were identified and partially characterized. As the major oligodN binding proteins on the plasma membranes of four different cell lines, these two proteins may play an important role in oligodN uptake, and thus constitute a major determinant of the intracellular action of oligodNs. The biological function of these proteins and the significance of binding of oligodNs and DNA on cell membranes remain to be determined. Purification, further characterization of these oligodN binding proteins, and the specificity of the binding of oligodNs are under study.

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