Dynamics of the Internalization of Phosphodiester Oligodeoxynucleotides in HL60 Cells†

C. A. Stein,* John L. Tonkinson, Li-Ming Zhang, Leonid Yakubov, James Gervasoni, Robert Taub, and Susan A. Rotenberg

Department of Medicine, Columbia University, 630 West 168 Street, New York, New York 10032, Institute of Bioorganic Chemistry, Novosibirsk, 640090, Russia, and Department of Chemistry and Biochemistry, Queens College, City University of New York, Flushing, New York 11335

Received June 3, 1992; Revised Manuscript Received January 20, 1993

ABSTRACT: We have examined the cellular association and internalization of phosphodiester (PO) oligodeoxynucleotides (oligos) with HL60 cells. At 4 °C, a 15-mer PO homopolymer of thymidine (FOdT15) exhibits apparent saturation binding ($K_m = 22 \pm 1$ nM) that is competitive with the binding of phosphorothioate (PS) oligos. The value of K_c for SdC28, a PS 28-mer homopolymer of cytidine, is 5 ± 2 nM. SdC28 was used to strip cell surface fluorescence: Internalized fluorescence accumulated in a (concentration)(time)-dependent fashion, consistent with a pinocytotic mechanism. PS, and to a lesser extent, PO oligos inhibited the rate of internalization of fluorescent albumin, also a marker of pinocytosis. This was correlated with direct in vitro inhibition of protein kinase C (PKC) β1 by the PS and PO oligos. Furthermore, other PKC inhibitors (H7, staurosporine, DMSO, PKC pseudosubstrate polypeptide) also inhibited intracellular accumulation of pinocytosed materials, perhaps by stimulating the exocytosis rate. In HL60 cells, the pinocytotic internalization of charged oligos appears to be dependent on intact PKC kinase activity, which is inhibited in vitro by PS and PO oligos.

Synthetic oligodeoxynucleotides (oligos) have been utilized as antisense inhibitors of mRNA translation in vitro (Stein & Cohen, 1988a; Zon, 1988; Helene & Toulme, 1990; Calabretta, 1991) and in vivo (Burch & Mahan, 1991; Simons & Rosenberg, 1992). They are also being evaluated for use as clinical therapeutic agents in human malignancies (Bayever, 1992). Because many classes of oligodeoxynucleotides [e.g., phosphodiesters (PO) and phosphorothioates (PS) (Stec et al., 1984; Stein & Cohen, 1988b)] are polyanions, they cannot passively diffuse through lipophilic cell membranes. On the other hand, the existence of cell membrane-bound DNA has long been recognized. Bennett et al. (1985) discovered a 30-kDa protein on peripheral blood lymphocytes that binds double-stranded DNA with $K_d = 1$ nM. The binding of DNA to the cell membrane was not inhibited by RNA, poly(dA–dT), oligodeoxynucleotides, or mononucleotides, but was inhibited by heparin. Loke et al. (1989), using homopolymers of thymidine with an acridine moiety covalently linked to the 5′-terminus, demonstrated that the cell-surface association of oligos with HL60 cells could be inhibited by other PO and PS homopolymers, 5′-nucleotide monophosphates, and tRNA, but not by heparin, ribose 5-phosphate, or uncharged thymidine methylphosphonate oligos. They isolated an 80-kDa cell-surface protein (p80) which appeared to be responsible for specific oligomer binding to the HL60 cells. Yakubov et al. (1989), in both L929 and Krebs ascite cells, also identified cell-surface proteins (of molecular masses 79 and 90 kDa) that could bind to PO oligos. However, these authors felt that the majority of oligo internalization was not due to receptor-mediated endocytosis, but rather was due to pinocytosis (fluid-phase endocytosis).

In the following experiments, we studied the dynamics of internalization of PO oligos in HL60 cells. We confirm that bulk internalization results predominately from pinocytosis. We also suggest that, in HL60 cells, the net cellular accumulation of oligo may be influenced by the activity of protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent protein kinase. PKC phosphorylates intracellular substrates on serine and threonine residues and is the major intracellular signal transduction protein (Rotenberg & Weinstein, 1991). Moreover, we also show that PS, and to a lesser extent, PO oligos of appropriate chain lengths can alter the kinase activity of PKC in vitro and that this correlates with the rates of intracellular internalization of some fluid-phase markers.

MATERIALS AND METHODS

Reagents. Phorbol 12-myristate 13-acetate was purchased from LC Services, (Woburn, MA). PKC pseudosubstrate peptide (R-F-A-R-K-G-A-L-R-Q-K-N-V-H-E-V-K-N) was purchased from Peninsula Laboratories (Belmont, CA), and H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride] was purchased from Seikagaku (Tokyo, Japan). Both were used without further purification. Staurosporine, thrombin, and bovine albumin were obtained from Sigma (St. Louis, MO). DMSO was obtained from Fisher (Pittsburgh, PA). Phosphatidylserine (PtdSer) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL), and [γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear (3000 Ci/mmol). The EGF receptor octapeptide was synthesized by N. Pileggi of the Protein Core Facility of Columbia University.

Synthesis of Oligodeoxynucleotides and Fluoresceination Procedure. All oligomers were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer using standard phosphoramidite technology as described by the manufacturer. After cleavage from the controlled pore glass

† This work was supported in part by grants (to S.A.R.) from the PSC-CUNY Research Award Program and by a grant to Queens College from the General Research Support Branch, NIH (RR-07064). Generous support (to C.A.S.) from the Columbia University Comprehensive Cancer Center is gratefully acknowledged.

* Author to whom correspondence should be addressed at Columbia University.

0006-2960/93/0432-4855$04.00/0

support by aqueous ammonia, oligo samples were base-deblocked in concentrated ammonia at 60 °C for 8 h. After purification by reversed-phase HPLC (0.1 M triethylammonium bicarbonate/acetonitrile, PRP-1 support), samples were detritylated in 3% acetic acid and precipitated with 2% lithium perchlorate/acetone. Concentrations of lithium as high as 1 mM had no effect on the internalization experiments as described below. PS oligos were synthesized by using the TETD (Applied Biosystems) reagent according to the manufacturer's instructions or by using the Beaucage reagent (Iyer, et al., 1990; generous gift of S. Beaucage).

For most flow cytometry experiments described below, we utilized, as the marker oligo, a 5′-fluorescein-labeled PO 15-mer homopolymer of thymidine, 5′-FITC-NH$(CH_2)_6OPO_3$-dT15-OH-3′, referred hitherto as FOdT15. In general, fluoresceinated oligodeoxynucleotides were synthesized after coupling of the 5′-terminus with Aminolink 2 (Applied Biosystems). After cleavage from the column and base deblocking as described above, the sample was lyophilized. To it was added 400 μL of sodium carbonate, pH 9.5, and 2 mg of fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, OR) dissolved in 80 μL of DMSO. The mixture was allowed to sit at room temperature for 16 h. For oligos greater than a 10-mer in length: The samples were dialyzed (1000 MW cutoff) against 0.5 M NaCl, 1 mM EDTA, and 0.1 M TEAB × 2 changes, omitting the NaCl and EDTA on the second change, followed by HPLC (PRP-1 support, 0.1 M TEAB (pH 8)/acetonitrile, increase acetonitrile 1%/min to 25 min). The retention time for FOdT15 is 19.3 min, as compared to 15.5 min for the 5′-amino-modified starting material. The oligomer was then further purified by electrophoresis on a 7 M urea/20% polyacrylamide gel. The yellow, fluorescein-labeled oligo band, which characteristically appeared between the bromophenol blue and xylene cyanole markers, was excised with a scalpel, crushed, and soaked in 0.1 M TEAB, and the eluate was passed through a column of Sephadex G-25 (Pharmacia). This procedure removes the last traces of free fluorescein. The final product was dissolved in a minimum volume of water and precipitated with 2% lithium perchlorate/acetone. For oligos of <10-mer in length, the dialysis step was omitted from the purification procedure. Oligomer concentrations were determined by UV spectroscopy.

Synthesis of Fluoresceinated Albumin. Bovine albumin (5 mg) (Sigma) was dissolved in 400 μL of sodium carbonate, pH 9.5. FITC (2 mg) dissolved in 80 μL of DMSO was added, and the mixture was allowed to sit at room temperature overnight. The volume of the mixture was then increased to 5 mL with water, the solution was dialyzed against 2 L of a solution of 0.5 M NaCl, 1 mM EDTA, and 0.1 M TEAB for 24 h, and then it was dialyzed against 0.1 M TEAB for an additional 12 h. The purity of the fluoresceinated albumin product was confirmed on a 20% polyacrylamide gel, and final protein concentration was determined by the Bradford protein assay (Bio-Rad Laboratories).

Cells. HL60 cells were grown in RPMI 1640/10% fetal calf serum (Gibco) to which was added 1 mM pyruvate, 2 mM glutamine, 100 mg/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM MEM nonessential amino acids. The fetal calf serum was heat-inactivated to 65 °C for 1 h before use. To test whether our serum contained nuclease activity, FOdT15 was incubated with heat-inactivated serum for time periods of up to 24 h. At all time points, only the original product, FOdT15, and no shorter fluorescent products was observed on 7 M urea/20% polyacrylamide gels after electrophoresis. Thus, our serum contained little, if any, 3′-exo- or 3′-endonucleolytic activity. For studies on the interaction of oligos with cells, 100 000 HL60 cells were treated with oligo (e.g., FOdT15) for the stated times and at the stated concentrations in 96-well microtiter plates. For cell-surface binding studies, cells were incubated with oligo at 4 °C. After the cells were washed to remove unbound fluorescence, mean cellular fluorescence intensities (average log channel for 10 000 observations, in triplicate, converted to linear fluorescence) were determined by a Becton-Dickenson FACStar flow cytometer and analyzed with either Consort 40 or DESK software.

Confocal Microscopy of HL60 Cells Treated with Oligos. HL60 cells incubated with fluoresceinated oligos (e.g., FOdT15) at the stated concentrations for the appropriate time periods were examined, without cellular fixation, using the MRC-600 laser-assisted confocal microscopy coupled to the 100× objective of a Leitz Ortholux II microscope as described previously (Gervasoni et al., 1991). The cells were imaged using the 488-nm line of the argon laser in conjunction with a 510-nm barrier filter. The digital images were stored in a computer and reproduced using a SONY videoprinter.

Inhibition of Protein Kinase C Activity by Oligos. In order to determine whether the in vitro activity of the PKC was inhibited by oligos, the β1 isoform was isolated from murine embryo C3H10T1/2 fibroblasts that had been genetically engineered to overproduce rat brain PKC-β1 (Krauss et al., 1989). The properties of this isoenzyme conform with those ascribed to native PKC isolated from rat brain (Rotenberg et al., 1990a). Details of its isolation have been reported elsewhere (Rotenberg et al., 1990b). Preparation of the enzyme used for the studies described here entailed elution of cell lysate material from DEAE-Sephacel. PKC activity was taken as the difference in the amount of [^{32}P]P_i transferred from [γ-^{32}P]ATP to a synthetic peptide sequence of the EGF receptor, R-K-R-T-L-R-R-L, in the absence and presence of PtdSer, as previously described (Housey et al., 1988; Rotenberg et al., 1990b).

The reaction medium (60 μL), placed in 13 × 100 mm disposable glass test tubes, consisted of 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1.0 mM $CaCl_2$, 2.0 μg of synthetic peptide (EGF receptor), 5 μg of PtdSer or 5 μL of water, 5 μL of approximately 0.5 mM oligo to be tested, and 5 μL of a PKC-β1 preparation (activity of 4–6 nmol of ^{32}P transferred/min/mL); the reaction was initiated with the addition of 5 μL of 792 μM [γ-^{32}P]ATP (ca. 200 cpm/pmol). Each (–) and (+) PtdSer condition was carried out in triplicate for each oligonucleotide concentration measured. The reaction was carried out in a 30 °C water bath for 10 min and was quenched by the transfer of 30 μL of the reaction medium to a 3 × 3 cm square of phosphocellulose paper and immediate immersion in a 1-L beaker of tap water. After all reactions were terminated, the squares were washed 5 times with 1 L of water and counted. Triplicate results were averaged and values were typically within 10–15% of the mean. The difference between the (+) PtdSer and the (–) PtdSer tubes was judged to be Ca^{2+}- and PtdSer-dependent activity. In control experiments, the presence of PtdSer typically stimulated the kinase reaction 4–7-fold with the synthetic EGFR peptide as substrate.

Determination of Competition Constants (K_c) of Competitors of FOdT15 Binding to the Surface of HL60 Cells. This was determined by the method of Cheng and Prusoff (1973), as given in eq 1. K_m is the Michaelis constant of

$$IC_{50} = K_c(1 + [FOdT15]/K_m) \quad (1)$$

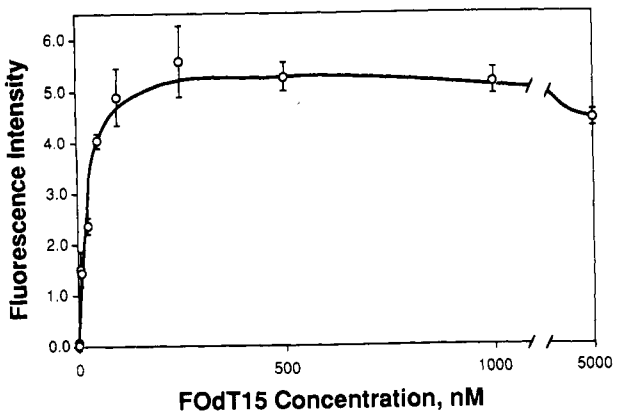

FIGURE 1: Titration of HL60 cells with FOdT15. FOdT15 at the indicated concentrations was added to HL60 cells in RPMI 1640/10% FBS at 4 °C for 5 min. The cells were washed twice with PBS/BSA and analyzed by a Becton-Dickenson FACStar Plus flow cytometer. Linear mean fluoresence intensities (mean log channel for 10^4 live cells converted to linear fluorescence) were determined. The experiments at each concentration were performed in triplicate. The value of K_m as determined from the curve is 22 nM.

binding of FOdT15 to the cell surface of HL60 cells. The oligo binds, presumably, to p80 as mentioned above. Data were collected on a FACS scanner: The value of IC_{50} was determined by titrating 100 000 HL60 cells with putative competitors (usually PS oligos) of FOdT15 binding to the cell surface of HL60 cells in the presence of a constant concentration of FOdT15. Data points were fit to a straight line by the method of linear least squares.

If the determination of K_c values for many competitors of oligo binding to the cell surface of HL60 cells is required, the use of eq 1 is cumbersome because the determination of IC_{50} requires large numbers of data points. Thus, in order to determine the values of K_c for large numbers of inhibitors, we have derived a modification of eq 1. If the assumption is made that the Michaelis constant, K_m, is approximately equal to K_D, then by definition, $K_D = K_m = (L_1)(R)/(L_1R)$, where L_1 = [FOdT15], R = [oligo receptor (p80)], and L_1R = concentration of the FOdT15/p80 complex. Thus, $K_c = (C)(R)/(R_c)$, where C = [competitor] and R_c = the concentration of the competitor-occupied receptor (p80). However, $L_1R + R_c = R_0$, where R_0 is the original concentration of unoccupied oligo binding sites, at saturating concentrations of FOdT15 and competitor. After solution for R_c and substitution, the result is

$$K_c/K_m = (C)/[\text{FOdT15}][1/((R_0/R_c) - 1)] \qquad (2)$$

R_0/R_c is the ratio of the mean cellular fluorescence channel numbers, as measured by FACS analysis, in the absence and presence of competitor, respectively. If [FOdT15] $\gg K_c$, then at IC_{50}, eq 2 is equivalent to eq 1.

RESULTS

Determination of K_m for FOdT15 and K_c for Competitors of Binding to HL60 Cells. In order to determine the values of K_c for competitors of FOdT15 binding to the surface of HL60 cells (presumably to p80), the value of K_m for FOdT15 binding was determined initially. This was done by adding increasing concentrations of FOdT15 to 10^5 HL60 cells (4 °C) in media. The mean channel fluorescence of FOdT15 bound to the HL60 cells vs [FOdT15] is shown in Figure 1. Data were obtained by FACS analysis as described above. The shape of the curve indicates that the binding to the cell surface is saturable, and the Lineweaver–Burk replot of these

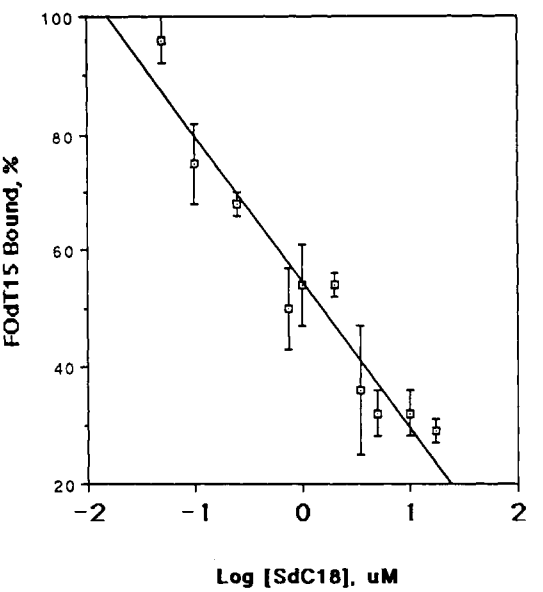

FIGURE 2: Concentration-dependent competition of cell-surface binding of labeled oligo by SdC18. FOdT15 (5 μM) was added to HL60 cells at 4 °C. Competition was performed with the indicated concentrations of SdC18 (4 °C, 3 min). Cells were then washed twice with PBS/BSA, and the relative surface-bound fluorescence was determined by FACS. The data was fit to a straight line by the method of linear least squares. Each point represents the average of the linear mean fluorescence of triplicate measurements, each representing 10^4 cells. Error bars represent standard deviations of the triplicates. The value of K_c was determined from the IC_{50} value and eq 1, as described in the text.

data (1/mean channel fluorescence vs 1/[FOdT15]) is linear ($R^2 = 0.97$). Thus, the association of FOdT15 with the surface of HL60 cells best fits a one-site binding model: This is consistent with a previous report (Loke et al., 1989) that a single species, presumably p80, is responsible for high-affinity oligo binding to the membrane of HL60 cells. The value of K_m may be calculated from Figure 1, or from the Lineweaver-Burk replot, as 22 ± 1 nM. The presence of a low-affinity binding site on HL60 cells for FOdT15 cannot conclusively be ruled out by these experiments, but on the basis of our preliminary results (C. Stein and T. Munir, unpublished results), if such a low-affinity site exists, the value of K_m must be $\gg 5$ μM.

We then examined a series of PS homopolymers of cytidine for their ability to act as competitors of FOdT15 binding to the surface of HL60 cells. Included in this series were SdC5, -8, -10, -12, -18, -21, and -28. The value of K_c for SdC18 was calculated from eq 1, after we determined the value of IC_{50} for competition. This determination is shown in Figure 2. The value of K_c for SdC18 could also be determined by use of eq 2, where [FOdT15] = 5 μM and C = [SdC18] = 1 μM; the value of K_c = 6 nM was determined by use of both eqs 1 and 2, thus demonstrating their equivalence. Table I shows the values of K_c for many PS and PO competitors of FOdT15 binding to the surface of HL60 cells. In these cases, K_c values were determined by eq 2, and experiments with each competitor were performed at least in triplicate.

Determination of Kinetic Parameters of the Association of FOdT15 with HL60 Cells. We used FACS analysis to measure the rate of loss of bound FOdT15 from the HL60 cells. Cells (10^5) were incubated at 4 °C with 5 μM FOdT15 for 30 min, washed twice with complete media, and then warmed to 37 °C. Cell-associated fluorescence was then monitored periodically by FACS. At this temperature, the rate of loss of FOdT15 from the cell surface followed a linear

Table I: Competition Constants (K_c) of Various Oligos for the Surface of HL60 Cells[a]

PS oligomer	K_c (nM)	PO oligomer	K_c (nM)
SdC5	308 ± 66	OdC5	162
SdC8	50 ± 26	OdC8	194
SdC10	38 ± 16		
SdC12	28 ± 18		
SdC15	69 ± 25	OdC15	78
SdC18	6 ± 1		
SdC21	9 ± 6	OdC21	16
OdC28	5 ± 2	OdC28	11

[a] FOdT15 and a nonlabeled competitor oligo were added to 1×10^5 HL60 cells at 4 °C for 5 min. The cells were washed twice with PBS/BSA, and the relative mean fluorescence for a population of 10^4 live cells was determined on a Becton-Dickenson FACStar Plus flow cytometer. (Mean log channel was converted to linear fluorescence by DESK software.) The competition constant (K_c) of the competitor could then be determined by use of either eq 1 or eq 2 (see text). PS K_c values represent the average K_c as determined in three independent experiments, each being the mean of triplicate determinations.

pseudo-first-order kinetic pathway for approximately 8 h (R^2 = 0.97). The finding of pseudo-first-order loss of FOdT15 from the HL60 cells is consistent with the hypothesis that loss of fluorescent oligo is, in fact, occurring at the cell surface, and not occurring by a process of oligo internalization followed by exocytosis. This is because we have observed that the exocytosis of internalized oligo is not log–linear and appears to best fit a multicompartment model (C. Stein and J. Tonkinson, unpublished results).

The value of k_{off}, the pseudo-first-order rate constant for the loss of FOdT15 from the surface of HL60 cells, is 3.1×10^{-3} min^{-1} ($t_{1/2}$ = 3.7 h). Thus, as $K_m = K_D = k_{off}/k_{on}$, $k_{on} = 1.4 \times 10^{-4}$ nM^{-1} min^{-1}. As $k_{obsd} = k_{on}$[FOdT15] (neglecting the contribution of k_{off}), at [FOdT15] = 5 μM $t_{1/2,on}$ = 1.0 min. Thus, all cell-surface binding sites are saturated rapidly by FOdT15 at a 5 μM concentration.

The number of cell-surface binding sites was determined by incubating 1×10^6 HL60 cells at 4 °C with increasing concentrations of 5′-OPO_3-OdT15 containing tracer amounts of 5′-$O^{32}PO_3$-OdT15. Cells were spun through a cushion of 1:1 dibutyl/dioctyl phthalate and counted. At saturation binding concentrations of 5′-OPO_3-OdT15 (1–5 μM), there were approximately (2–4) $\times 10^5$ binding sites/cell. However, because of the greatly increased ability of dead cells and debris to associate with oligos, this value represents only an upper limit of the number of oligo binding sites/cell.

The binding of PO oligos to the surface of HL60 cells is Ca^{2+} (but not Mg^{2+} or SO_4^{2-}) dependent. In Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4), FOdT15 binding is only 30% of the maximal binding, which is achieved in PBS at 100 μM Ca^{2+}. On the other hand, the binding of FSdT15 (the PS analog of FOdT15) to the cell surface of HL60 cells is not Ca^{2+} dependent, and Cd^{2+} (10 μM), which will precipitate FSdT15 from solution, cannot strip it from the cell surface.

Internalization of FOdT15 in HL60 Cells. In Figure 3, we show the relationship between the concentration and time dependence of FOdT15 association with HL60 cells. Three concentrations of FOdT15 were used, 0.15, 1, and 5 μM, all of which greatly exceed the value of K_m (22 nM). The monotonic increase in cellular association with increasing concentration and time is shown in Figure 3 (top). However, analysis of the mechanism of oligo internalization is complicated by the difficulty in distinguishing internalized from membrane-bound oligomers. Because we had determined the

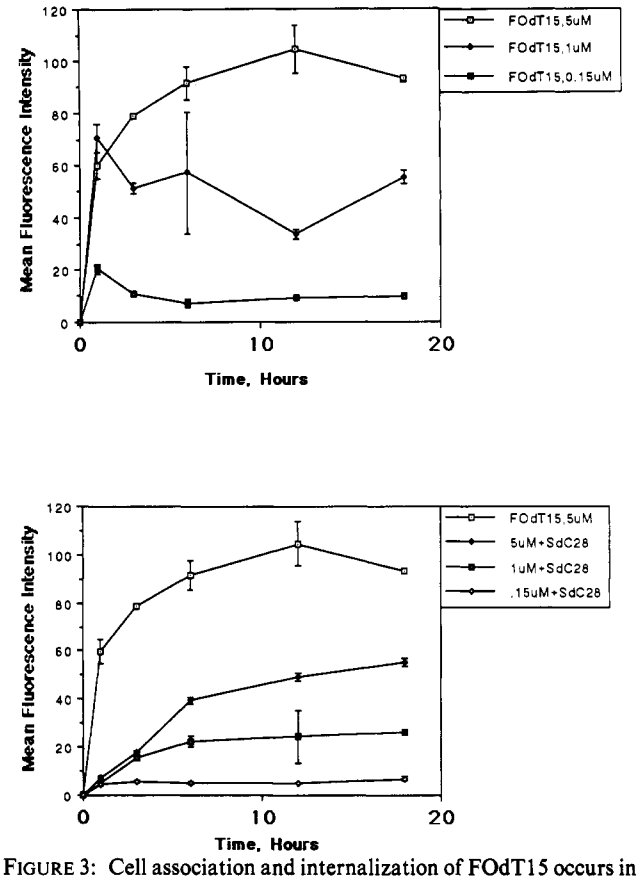

FIGURE 3: Cell association and internalization of FOdT15 occurs in a (concentration)(time)-dependent manner. Top panel: HL60 cells were incubated at 37 °C (RPMI 1640/10% FBS) with 0.15, 1, and 5 μM oligomer for the indicated times and washed twice with PBS/BSA. Relative mean fluorescence intensities were determined by FACS analysis. Botton panel: Prior to washing, SdC28 (5 μM) was added to the wells to remove all surface-bound label (see text). The cells were then prepared for FACS analysis as previously described. Error bars represent standard deviations for the linear mean fluorescence values for triplicate measurements.

values of K_c for competitors of FOdT15 binding to HL60 cells, we were aware of the approximately 1-log difference between the K_m of FOdT15 (22 nM) and the K_c of SdC28 (3 nM). We used this difference in employing SdC28 to strip off all of the bound FOdT15 from the cell surface; the remaining cell-associated fluorescence was then internalized. This process eliminated the necessity for acid washing, which in our hands did not give reproducible results. In Figure 3 (bottom), 10^5 HL60 cells, after treatment with either 0.15, 1, or 5 μM FOdT15 for the indicated times, were washed with PBS/BSA (pH 7.4) to remove unbound FOdT15 and treated with 5 μM SdC28 for approximately 5 min. FACS analysis was then performed. Significantly, here too, internalization as well as cellular association proceeded as a monotonic function of (concentration)(time). This finding suggests that pinocytosis accounts for the majority of cellular internalization of FOdT15 in HL60 cells, at least at the concentration of FOdT15 we employed. It is not likely that adsorptive endocytosis plays a major role in internalization, at least not at [FOdT15] > 22 nM, because the rate of internalization as a function of (concentration)(time) does not plateau above K_m.

The ability of SdC28 to quantitatively remove cell-surface-bound fluorescence was demonstrated by confocal microscopy. At 3 and 6 h of incubation, FOdT15 could be seen bound only to the cell membrane: virtually no internalized fluorescence could be detected. After addition of SdC28 (1 or 5 μM), all

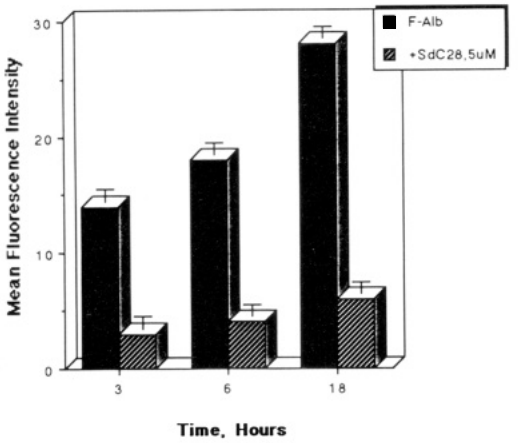

FIGURE 4: SdC28: a potent blocker of cellular uptake of fluorescent albumin. HL60 cells were incubated at 37 °C (RPMI 1640/10% FBS) with 0.1 mg/mL fluorescein-labeled albumin added for the indicated times. Where indicated, SdC28 (5 μM) was added with the albumin. Cells were washed twice and prepared for FACS analysis. Linear mean fluorescence was determined for 10^4 cells per well, with each treatment being performed in triplicate.

membrane-bound fluorescence was removed. At 18 h, internalized fluorescence, in a patchy or punctate pattern, was observed. Intranuclear staining was virtually nil at all times.

When FOdT15 was incubated for longer time periods (12–24 h) with the HL60 cells, as is seen in Figure 3, a decrease in the total cell–associated fluorescence was noted. However, this decrease coincided with increased intracellular accumulation of fluorescence. We hypothesized that this could indicate that the number of cell-surface oligo binding sites was decreasing as a function of the time of incubation. In order to examine this, we pulsed 10^5 HL60 cells in complete media with OdT15 (K_c = 55 nM, 5 μM) for 18 h of incubation, washed the cells (4 °C), and then probed the number of cell-surface binding sites by treating the cells with FSdT15 (K_c = 0.2 nM), the PS analog of FOdT15 (10 μM, 4 °C). We then quantified, by FACS, the amount of cell-surface fluorescence: The number of cell-surface oligo binding sites in the OdT15-treated cells, as compared to control cells not pulsed with OdT15, consistently declined by approximately 40%.

Oligodeoxynucleotides Affect the Pinocytosis Rate in HL60 Cells. We have used fluorescently labeled albumin (F-albumin, 0.05–0.1 mg/mL in complete media) in order to determine how oligos and other substances affected the rate of oligo internalization, which, as described above, appears to occur predominately via the process of pinocytosis. F-albumin, in HL60 cells, is internalized in a (concentration)(time)-dependent manner (data not shown) and is thus a good marker for fluid-phase endocytosis. In Figure 4, we show the effects of a PS oligo on the rate of F-albumin internalization. At all time points shown (3, 6, and 18 h), SdC28 (5 μM) is a potent inhibitor (20% of control fluorescence) of F-albumin internalization. At this concentration, SdC28 exhibits no cellular cytotoxicity, as judged by cellular growth curves and propidium iodide staining. Furthermore, a PO oligomer, OdC28 (5 μM), inhibits F-albumin internalization (32% of control fluorescence value) after 6 h of incubation, whereas OdC5 is ineffective. SdC11 (5 μM) also inhibits internalization (32% of control value, 6 h). Other polyanions, including dextran sulfate (500 kDa, 1 mg/mL, 27% of control value) and suramin (75 μg/mL, 37% of control value), can also inhibit F-albumin internalization. Similar types of results were observed as well for the polyanions aurintricarboxylic acid and pentosan polysulfate (polyxylyl hydrogen sulfate).

SdC28 Affects the Exocytosis Rate of Fluorescent Oligonucleotides and Fluorescent Albumin but not Sucrose. We wanted to determine whether SdC28 could alter the net accumulation of pinocytosed material by affecting the endocytosis rate, the exocytosis rate, or both. In conjunction with endocytosis experiments performed, with fluoresceinated oligos and F-albumin, both markers of pinocytosis, we have also examined the internalization of sucrose (Amersham) by HL60 cells. Sucrose is not metabolized, is membrane impermeable, and has long been recognized as a fluid-phase marker (Besterman et al., 1981). Experiments examining the rate of sucrose internalization in HL60 cells were performed in 1 mM sucrose with tracer amounts of [^{3}H]sucrose added. Our data indicated that sucrose, as has been previously described (Besterman et al., 1981), was internalized in HL60 cells via a (concentration)(time)-dependent pathway. Although incubation of HL60 cells with F-albumin and increasing concentrations of SdC28 caused a dose-dependent decrease in F-albumin endocytosis at 6 h (IC_{50} = 1 μM), the same concentrations of SdC28 caused no change in the rate of cellular accumulation of [^{3}H]sucrose at 6 h, as compared to control cells in the absence of SdC28. We then asked whether SdC28 exerted its effects predominately upon the rate of exocytosis of sucrose. However, the rate of [^{3}H]sucrose efflux from HL60 cells was not changed even at the highest concentrations of SdC28 (20 μM) tested. This observation is consistent with the hypothesis that the intracellular trafficking of the small, neutral sucrose molecule differs from that of a charged oligonucleotide or of F-albumin, even though both may be internalized in the fluid phase.

Data supporting this interpretation was obtained by incubating HL60 cells for 6 h in the presence of F-albumin, washing the cells, and then adding SdC28 (5 μM) 1 h before quantitation of cellular fluorescence by FACS analysis. The addition of SdC28 consistently led to a 40% decrease in intracellular fluorescence relative to the control samples, to which no SdC28 was added. Similar results were obtained using FOdT15 in place of F-albumin. These data suggest that SdC28 affects the exocytosis rate of FOdT15 and F-albumin and that an increase in the exocytosis rate is responsible for net decreased internalization of these fluid-phase markers.

Inhibitors of Protein Kinase C (PKC) Affect the Rate of Internalization of FOdT15 and F-Albumin. As is shown in Figure 5, several inhibitors of PKC activity are, when coincubated with FOdT15 (5 μM) for 12 h, capable of diminishing the cellular association of FOdT15 with HL60 cells. These inhibitors include H7 (100 μM), 1% DMSO, the PKC pseudosubstrate oligopeptide (50 μM), and staurosporine (100 nM, in ethanol, data not shown). We have verified that the amount of internalized oligo also decreases in the presence of PKC inhibitors vs control (no PKC inhibitor). To show this, we attempted to remove cell-surface-bound oligomer with SdC28. However, in the presence of PKC inhibitors, SdC28 did not remove any FOdT15 from the cell surface; this indicates that in the presence of the PKC inhibitors, all of the oligo is internalized and the cell-surface oligo binding sites have been lost.

It is possible that the data obtained on the internalization of F-albumin or FOdT15 in the presence of PKC inhibitors could result from fluorescence quenching, which could occur in an acidic intracellular compartment. In order to eliminate this possibility, we examined the internalization of lissamine-rhodamine albumin (LR-albumin), whose fluorescence emis-

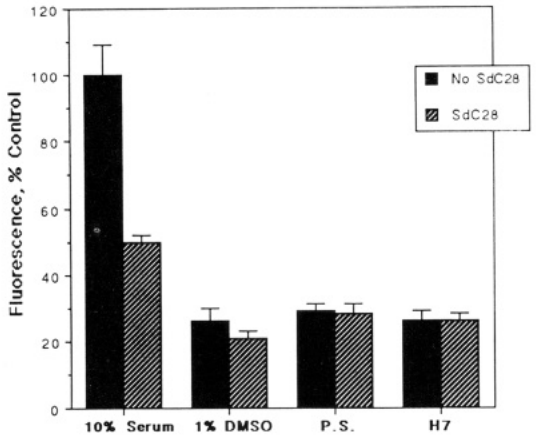

FIGURE 5: Protein kinase C inhibitors decreasing the cellular association of FOdT15. HL60 cells were incubated overnight in RPMI 1640/10% FBS at 37 °C, alone or with the PKC inhibitors 1% DMSO, PKC pseudosubstrate peptide (50 μM), or H7 (100 μM). FOdT15 (5 μM) was added over 6 h at 37 °C. Where indicated, SdC28 (5 μM) was added at 4 °C over 3 min immediately prior to preparation for FACS analysis. Linear mean fluorescence was determined for 10^4 cells, with each treatment being performed in triplicate.

sion is not pH-sensitive. Both SdC28 (5 μM) and H7 (100 μM), under identical experimental conditions as used previously (coincubation with LR-albumin, 1 mg/mL, 12 h), were dramatic inhibitors of LR-albumin internalization, as measured by FACS. Furthermore, we collapsed the pH gradient across the endosomal membrane by adding monensin (6 μM; Maxfield, 1982) to cells that had been loaded with FOdT15 (5 μM) for varying amounts of time. No increase in fluorescence emission was noted at any time (1–24 h), indicating that the fluorescence label never appears to reside in an acidic endocytic compartment. The control, F-albumin-loaded cells, both in the presence and absence of SdC28, however, showed a dramatic increase in fluorescence after monensin treatment.

On the other hand, stimulators of PKC activity appeared to increase FOdT15 internalization. After 6 h of incubation, PKC activity is up-regulated in HL60 cells by PMA/ethanol (Tran et al., 1989). When HL60 cells were incubated with 25 nM phorbol 12-myristate 13-acetate (PMA) for 6 h, the cell association of FOdT15 was increased by over 150% relative to the control. PMA treatment could also partially overcome the inhibitory effects of 1% DMSO treatment. On the other hand, at 24 h, when PKC activity is down-regulated by PMA, no effect on the cell association of FOdT15 was seen. Thrombin (0.5 mg/mL), also a stimulator of PKC activity (Herrick-Davis et al., 1991), increased (by 20%) the cell association of FOdT15 as well.

Oligodeoxynucleotides Are Potent, Direct Inhibitors of PKC-β1 Activity. As shown in Figure 6, both PS and PO oligodeoxynucleotides are potent, concentration-dependent, non-sequence-specific inhibitors of PtdSer-stimulated PKC-β1 activity. The inhibition is also highly chain length dependent and is greater at any given concentration for PS than for PO oligomers. For SdC28, the value of IC_{50} is approximately 1 μM. Inhibition of PKC-β1 activity by SdC28 thus coincides with the observation of decreased internalization of oligos and of albumin via fluid-phase endocytosis. Moreover, these data suggest that in HL60 cells even at low concentration SdC28, perhaps via its inhibitory effect on PKC activity, might be capable of interfering with its own internalization.

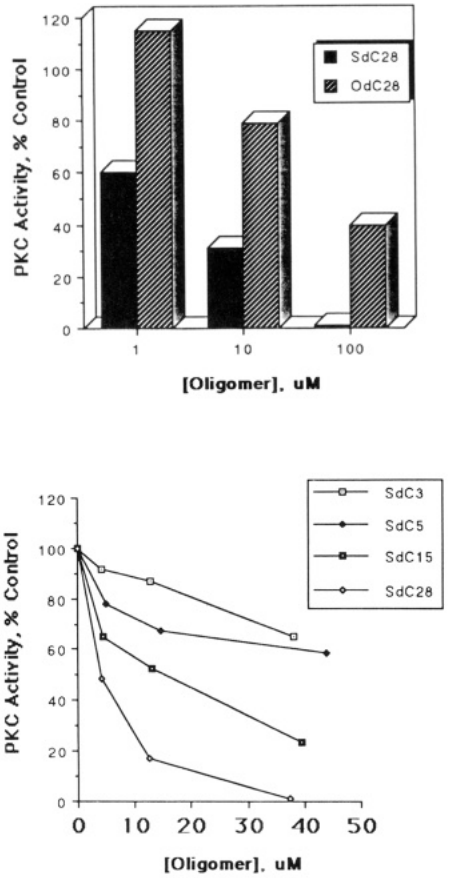

FIGURE 6: Oligodeoxynucleotide direct inhibition of protein kinase C. In vitro protein kinase C assays were performed with purified PKC-β1 as described in Materials and Methods. PS or PO oligos were added to the reaction mixture at the indicated concentrations. PKC kinase activity (^{32}P transferred from [γ-^{32}P]ATP to a synthetic peptide in the presence of PtdSer minus the amount transferred in the absence of PtdSer) was compared between samples with oligo and positive controls. Each experiment was performed in triplicate.

DISCUSSION

The data reported in the foregoing experiments were obtained using synthetic, fluorescent oligodeoxynucleotides that were free from FITC basic degradation products that could have introduced considerable irreproducibility into the data. In our hands, the best way to eliminate these contaminants was via 7 M urea/20% polyacryamide gel electrophoresis, followed by excision of the fluoresceinated oligo band. Furthermore, use of FACS analysis, rather than radioactive tracer methods, allowed for gating of dead cells and debris, both of which freely accumulate oligodeoxynucleotides in the same manner as they do other polyanions, such as trypan blue. Failure to perform gating can lead to large, irreproducible differences between the arithmetic means and modes of experimentally derived fluorescence intensities. However, when radioactive oligos were used, because of the potential for even slight contamination, we could only determine an upper limit for the number of cell-surface oligodeoxynucleotide binding sites. The striking length dependence of K_c on PS oligo chain length (Table I) is not unique to p80. We (Stein et al., 1993) have observed a similar dependence for the nonspecific binding of PS oligos to gp120, as have Gao et al. (1992) for PS oligo inhibition of RNAse H1. The common structural motifs that give rise to this pattern are not known.

While cell-surface-bound FOdT15 undergoes capping, this process takes hours and is much slower than the internalization rate for LDL (approximately 15 min), which is internalized predominately via receptor-mediated endocytosis (Goldstein

& Brown, 1976). Furthermore, the (concentration)(time) dependence of internalization, at concentrations several orders of magnitude higher than the experimental value of K_m (22 nM), suggests that fluid-phase endocytosis or pinocytosis is responsible for the bulk of internalized FOdT15.

On the basis of the data that we have obtained in our studies, we are able to propose a model that accounts for the internalization of PO oligos in HL60 cells. Our data suggest that the rate of internalization of fluorescently labeled PO oligomers may be controlled by affecting the exocytosis rate: long-chain PS oligos, e.g., SdC28, increase the exocytosis rate of some fluid-phase markers (FOdT15, F-albumin). The blocking effect of PS oligomers upon their own uptake may explain why several laboratories (Reed et al., 1990; Matsukura et al., 1989) have reported that many days are required for sequence-specific PS oligos to produce antisense effects, although it must be pointed out that in neither case were HL60 cells employed. Furthermore, the net rate of oligo internalization, which appears to depend on the pinocytosis rate, may itself be regulated by PKC kinase activity. Keller (1986) has shown that diacylglycerols and PMA stimulated the pinocytosis rate in human neutrophils. We have made similar observations after 6 h of treating HL60 cells with PMA; however, it should be noted that PMA will, after several days of exposure, differentiate HL60 cells into highly phagocytic macrophages. It is thus possible that our results with PMA are reflective of changes initiated at several points in the differentiation pathway. However, we have also noted that PKC inhibitors (e.g., H7, staurosporine, SdC28) inhibited the internalization of fluid-phase markers in HL60 cells. This is similar to what has been reported by Dewald et al. (1989), who found that the PKC inhibitor staurosporine induced exocytosis in human neutrophils. However, both PO and PS oligos themselves are PKC inhibitors. Thus, in HL60 cells, internalization, via fluid-phase endocytosis, of oligos of long (>10–15 mer) chain lengths may lead, via PKC inhibition and stimulation of exocytosis, to a diminished intracellular oligo concentration, which we observe as decreased fluorescence.

This model may be useful in understanding some other results: In an attempt to specifically inhibit PKC in HL60 cells, we targeted sequence-specific 15-mer PO oligos to an isoform-conserved region of the PKC mRNA. As the end point, we examined the internalization rate of F-albumin. However, both sense and antisense contructs were equally effective at reducing internalization. This may result from non-sequence-specific inhibition of PKC activity by the PO oligo and stimulation of exocytosis activity.

The model also lends some support to the contention of Shoji et al. (1991), who suggest that endosome to cytoplasm transfer of oligo (thus removing it from a compartment subject to rapid exocytosis under the influence of PKC) may be a critical factor in the pharmacological actions of charged oligos. However, detailed knowledge of oligomer efflux pathways is not yet available and is a subject of continuing, intense interest. Likewise, there are many other factors, apart from PKC kinase activity, that could have an impact on the rate and extent of oligo internalization that we have not yet identified. Furthermore, it is extremely important not to generalize from these results in HL60 cells to other cells either in tissue culture or in vivo. It is a matter of intense interest now whether the processes described above are limited to HL60 cells or whether they form a more general framework for describing oligo internalization. In order for oligos to be utilized successfully for therapeutic applications, it seems imperative to pursue a more detailed understanding of these mechanisms.

ACKNOWLEDGMENT

The authors gratefully acknowledge stimulating discussions with Drs. I. B. Weinstein and Alan Stall and thank Dr. G. Zon, Applied Biosystems, for the SdC28 used in these experiments. We also thank Dr. B. Dolnick for a critical review of the manuscript and the reviewers for insightful comments.

REFERENCES

Bayever, E. (1992) *Antisense Res. Dev. 2*, 109.
Bennett, R., Gabor, G., & Merritt, M. (1985) *J. Clin. Invest. 76*, 2182–2190.
Besterman, J., Airhart, J., Woodworth, R., et al. (1981) *J. Cell Biol. 91*, 716–727.
Burch, R., & Mahan, L. (1991) *J. Clin. Invest. 88*, 1190–1196.
Calabretta, B. (1991) *Cancer Res. 51*, 4505–4510.
Cheng, Y., & Prusoff, W. (1973) *Biochem. Pharmacol. 22*, 3099–3108.
Dewald, B., Thalen, M., Wymann, M., et al. (1989) *Biochem. J. 264*, 879–884.
Gao, W.-Y., Han, F., Storm, C., et al. (1992) *Mol. Pharmacol. 41*, 223–229.
Gervasoni, J., Fields, S., Krishna, S., et al. (1991) *Cancer Res. 51*, 4955–4963.
Goldstein, J., Basu, S., Brunschede, G., et al. (1976) *Cell 7*, 85–95.
Helene, C., & Toulme, J. (1990) *Biochim. Biophys. Acta 1049*, 99–125.
Herrick-Davis, K., Camussi, G., Bussolino, F., et al. (1991) *J. Biol. Chem. 266*, 18620–18625.
Housey, G., Johnson, M., Hsiao, W. L., et al. (1988) *Cell 52*, 343–354.
Iyer, R., Egan, W., Regan, J., et al. (1990) *J. Am. Chem. Soc. 112*, 1253–1254.
Keller, H. (1990) *J. Cell. Physiol. 145*, 465–471.
Krauss, R., Housey, G., Johnson, M., et al. (1989) *Oncogene 4*, 991–998.
Loke, S., Stein, C., Zhang, X., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3474–3478.
Matsukura, M., Shinozuka, K., Zon, G., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4244–4249.
Maxfield, F. (1982) *J. Cell Biol. 95*, 676–681.
Rotenberg, S., & Weinstein, I. B. (1991) in *Biochemical and Molecular Aspects of Selected Cancers* (Pretlow, T. G., & Pretlow, T. P., Eds.) pp 25–73, Academic Press, Orlando, FL.
Rotenberg, S., Krauss, R., Borner, C., et al. (1990a) *Biochem. J. 266*, 173–178.
Rotenberg, S., Smiley, S., Ueffing, M., et al. (1990b) *Cancer Res. 50*, 677–685.
Reed, J., Stein, C., Subasinghe, C., et al. (1990) *Cancer Res. 50*, 6565–6570.
Shoji, Y., Akhtar, S., Periasamy, A., et al. (1991) *Nucleic Acids Res. 19*, 5543–5550.
Stec, W., Zon, G., Egan, W., et al. (1984) *J. Am. Chem. Soc. 106*, 6077–6079.
Stein, C., & Cohen, J. (1988) *Cancer Res. 48*, 2659–2668.
Stein, C., Subasinghe, C., Morgan, N., et al. (1988) *Nucleic Acids Res. 16*, 3209–3221.
Stein, C., Cleary, A., Yakubov, L., & Lederman, S. (1993) *Antisense Res. Dev.* (in press).
Tran, P., LePeuch, C., & Basset, M. (1989) *J. Cell. Physiol. 139*, 313–319.
Yakubov, L., Deeva, E., Zarytova, V., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6454–6458.
Zon, G. (1988) *Pharm. Res. 5*, 539–549.