Interactions of Phosphodiester and Phosphorothioate Oligonucleotides with Intestinal Epithelial Caco-2 Cells

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Purpose. Oral bioavailability for antisense oligonucleotides has recently been reported but the mechanistic details are not known. The proposed oral delivery of nucleic acids will, therefore, require an understanding of the membrane binding interactions, cell uptake and transport of oligonucleotides across the human gastro-intestinal epithelium. In this initial study, we report on the cell-surface interactions of oligonucleotides with human intestinal cells.

Methods. We have used the Caco-2 cell line as an in vitro model of the human intestinal epithelium to investigate the membrane binding interactions of 20-mer phosphodiester (PO) and phosphorothioate (PS) oligonucleotides.

Results. The cellular association of both an internally [^{3}H]-labelled and a 5'end [^{32}P]-labelled PS oligonucleotide (3.0% at 0.4 μM extracellular concentration) was similar and was an order of magnitude greater than that of the 5'end [^{32}P]-labelled PO oligonucleotide (0.2%) after 15 minutes incubation in these intestinal cells. The cellular association of PS was highly saturable with association being reduced to 0.9% at 5 μM whereas that of PO was less susceptible to competition (0.2% at 5 μM, 0.1% at 200 μM). Differential temperature-dependence was demonstrated; PS interactions were temperature-independent whereas the cellular association of PO decreased by 75% from 37°C to 17°C. Cell association of oligonucleotides was length and pH-dependent. A decrease in pH from 7.2 to 5.0 resulted in a 2- to 3-fold increase in cell-association for both backbone types. This enhanced association was not due to changes in lipophilicity as the octanol:aqueous buffer distribution coefficients remained constant over this pH range. The ability of NaCl washes to remove surface-bound PS oligonucleotides in a concentration-dependent manner suggests their binding may involve ionic interactions at the cell surface. Cell-surface washing with the proteolytic enzyme, Pronase®, removed approximately 50% of the cell-associated oligonucleotide for both backbone types.

Conclusions. Binding to surface proteins seems a major pathway for binding and internalization for both oligonucleotide chemistries and appear consistent with receptor (binding protein)-mediated endocytosis. Whether this binding protein-mediated entry of oligonucleotides can result in efficient transepithelial transport, however, requires further study.

KEY WORDS: antisense oligonucleotides; drug delivery; cell binding; cellular uptake; intestinal cells; Caco-2 cells.

INTRODUCTION

The ability of antisense oligonucleotides to specifically hybridize with target mRNA sequences and cause translation arrest makes them attractive candidates for potential therapeutic applications (1). Although unmodified phosphodiester (PO) oligonucleotides are unlikely to be used clinically due to their poor biological stability (2), several phosphorothioate-modified (PS), oligonucleotides are undergoing clinical trials evaluation for the treatment of HIV-1 infection and cytomegalovirus retinitis in AIDS patients, human papillomavirus-infected viral warts, and acute and chronic myelogenous leukaemias [3].

Despite the rapid progress of oligonucleotides into clinical trials as potential therapeutic agents, efficient delivery into target cells remains a problem and a number of delivery strategies and routes of administration have been investigated (for a recent review, see ref. 4). Recently, oral delivery of oligonucleotides has been investigated in animals (5–7) and these reports, somewhat surprisingly, suggest that stable analogues may indeed be able to traverse the gastro-intestinal tract.

In an attempt to understand further the likely interactions of oligonucleotides with the gastro-intestinal epithelium, we have been investigating the binding, uptake and transepithelial transport of oligonucleotides. In this initial study, we report on the cell surface binding interactions of PO and PS oligonucleotides in cultured intestinal (Caco-2) cells. These cells were chosen because monolayers of these polarized, human colon carcinoma cell line are considered to be a suitable in vitro model for investigating intestinal drug uptake and transport (8–10). We report that PS oligonucleotides have a greater affinity for Caco-2 cell association than PO oligonucleotides. The affinity of oligonucleotides to bind to these intestinal epithelial cells can be further enhanced by increasing oligonucleotide length or by decreasing the pH of the surrounding environment. The increased binding does not appear to be due to increased partitioning of oligonucleotides into the lipid bilayer. Instead, cell association, and presumably internalization, of both oligonucleotides appears to involve increased ionic interactions with cell-surface binding proteins.

MATERIALS AND METHODS

Materials

All chemicals were cell culture grade or the highest purity available from Sigma Chemical Company (Poole, UK) unless otherwise specified. All cell culture media-components and reagents were purchased from Gibco (Paisley, UK). Radiochemicals were purchased from Amersham (Amersham, UK). Reagents for oligonucleotide syntheses were purchased from Applied Biosystems (Warrington, UK) and Cruachem (Glasgow, UK). All reagents were used as received without further purification.

Cell Culture

Two media were prepared for culturing Caco-2 cells. A maintenance-medium was used to maintain stock cultures in 150cm^2 plastic T-flasks and a plate medium was used to cultivate cells on 24-well plates. The Maintenance medium comprised Dulbecco's modification of Eagle's medium supplemented with (final concentrations) 10% v/v foetal bovine serum, 1% v/v non-essential amino acids and 1% v/v L-glutamine. Plate medium comprised maintenance medium supplemented to a final con-

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centration of 1% v/v with penicillin and streptomycin. Cells were incubated at 37°C in a humidified (95%) atmosphere of 10% CO_2 in air. Maintenance medium was renewed every 48 hours. Stock-cultures were passaged (1:3) weekly by trypsinisation with a solution containing 0.25% w/v trypsin and 0.2% w/v disodium ethylenediamine tetraacetate in phosphate buffered saline, pH 7.2 (PBS; Oxoid, UK). Cells were used for experiments between passages 85 and 110.

For uptake studies, Caco-2 monolayers were cultured on plastic 24-well plates (Costar, Cambridge, USA). Briefly, 2–3 day post-confluent stock-cultures were trypsinised and resuspended in plate medium. The viable cell-density was measured by haemocytometry using a trypan blue-exclusion test and reduced to 2.0×10^5 cells ml^{-1} by dilution with further plate medium. Each well was seeded with 1 ml of the diluted cell-suspension. The 24-well plates were incubated at 37°C in a humidified atmosphere (95%) of 10% CO_2 in air. Plate medium was renewed every second day and the monolayers used for uptake studies after 7 days in culture, the cell density having reached $\sim 2.5 \times 10^5$ cells $well^{-5}$.

Uptake Media

The media used for uptake studies comprised Hanks' balanced salt solution (HBSS) containing, 0.01% phenol red, 5 mM D-glucose and buffered with 25 mM HEPES (pH 7.0, 7.2, 7.4, and 8.0) or MES (pH 5.0, 5.5, 6.0, 6.5). They were filter-sterilized using a bottle-top filter system (Costar) and stored at 4°C.

Uptake Experiments

Plate medium was aspirated and the monolayer carefully washed with uptake medium (1×1 ml $\times$ 15 min). The uptake experiment was initiated by aspirating the washing-solution and replacing it with 200 µl of uptake medium containing the radiolabelled ligand of interest. Washing and incubation solutions were equilibrated to 37°C for 1 hour prior to use. The monolayers were placed in an incubator (37°C) unless otherwise stated, for the duration of the uptake experiment. After the incubation-period, monolayers were transferred to a cold table (0–4°C). The incubation solutions were collected and their radioactive content determined by liquid scintillation counting (LSC, Beckman, LS 1801). Each monolayer was carefully rinsed (4×0.5 ml $\times$ 5 min) with ice-cold PBS-sodium azide 0.05% w/v (PBS-N_3). Following the final PBS-N_3 wash, monolayers were detached by shaking (Dynatech, Micro shaker) with 0.5 ml of 0.1% v/v Triton X-100 (Aldrich Chemical Company, Gillingham, UK) in double-distilled water for 60 min at 25°C. The wells were washed with a further 3×0.5 ml of Triton X-100 which along with the previous 0.5 ml were collected for LSC. The cell-associated radioactivity was expressed as the percentage of the total applied to the monolayer (*i.e.* cells + washings + apical samples).

Post-uptake Washing Experiments

The oligonucleotide uptake experiment was performed as described above and then followed by post-uptake studies. For the sodium chloride wash experiment, the first PBS-N_3 wash was replaced with the appropriate ionic wash and the experiment was continued as before. For the pronase-wash experiment, the cells were incubated with the oligonucleotide as before and then washed with 4×0.5 ml PBS-N_3. The monolayers were then treated with 0.25% w/v pronase (Calbiochem, Nottingham, UK) in HBSS (pH 7.2 and 4°C) for 45 minutes and subsequently removed with Triton X-100 as before.

Octanol: Aqueous Buffer Distribution Coefficients

The distribution coefficients between n-octanol and aqueous (McIlvaines) buffer (0.1 M citric acid, 0.2 M disodium hydrogen phosphate) were determined over a pH range 2.7 to 8.6. Prior to distribution studies, the two immiscible phases were pre-saturated at 37°C by shaking (Gallenkamp orbital incubator, 100 rpm) for 24h. Trace amounts of radiolabelled oligonucleotide were then added to the phases and shaken for 3 hours. Samples from each phase were taken and counted using LSC. Data are expressed as the log distribution coefficient (log D) between the two phases thus:

$$\text{Log D} = \text{Log} \frac{[\text{Oligo}]_{\text{octanol}}}{[\text{Oligo}]_{\text{McIlvaines}}}$$

Synthesis, Labelling, and Purification of Tat Oligonucleotides

Antisense oligonucleotides, complementary to the 3′-splice site of the *tat* gene in HIV RNA, were synthesised on an Applied Biosystems 392 DNA Synthesiser. The 20-*tat* sequence (5′-GGT AAA AGT CTT AAC CCA CA-3′) was synthesised using standard phosphoramidite chemistry in PO and PS forms. ISIS3521, a 20mer PS oligonucleotide (5′-GTT CTC GCT GGT GAG TTT CA-3′), directed against the 3′ untranslated region of human PKCα mRNA, was a generous gift from Dr. Nicholas Dean (ISIS Pharmaceuticals, Carlsbad, USA).

Prior to use in uptake studies, the *tat* oligonucleotides were labelled at the 5′-end with ^{32}P, using polynucleotide T_4 kinase and [γ-^{32}P]ATP according to the manufacturers protocol (Gibco, Cat No. 8004SA). The resultant product was purified by 20% native polyacrylamide gel electrophoresis (PAGE) and the oligonucleotides were quantified using UV spectroscopy at 260 nm. [^{3}H]ISIS3521 was prepared using the protocol of Graham *et al.* [11] and purified using size-exclusion chromatography (Sephadex G10).

RESULTS

Oligonucleotide Stability and Cellular Association-time Profile

The cellular association (Fig. 1) and stability of oligonucleotides in the apical solutions above Caco-2 monolayers (Fig. 2) were defined at selected time intervals between 15 and 300 min. The cellular association of both a 5′ end [^{32}P]-labelled and an internally [^{3}H]-labelled PS oligonucleotides were investigated in Caco-2 cells at pH 7.2 and at 37°C. Cellular association of 5′ end labelled PS oligonucleotide appeared to occur via a biphasic process with an initial rapid phase of association over the first 15 minutes being followed by a slower secondary phase of cellular interaction. A similar profile was also observed for the [^{3}H]-labelled ISIS3521 (see inset to Figure 1), however, at the later time points, the cell-

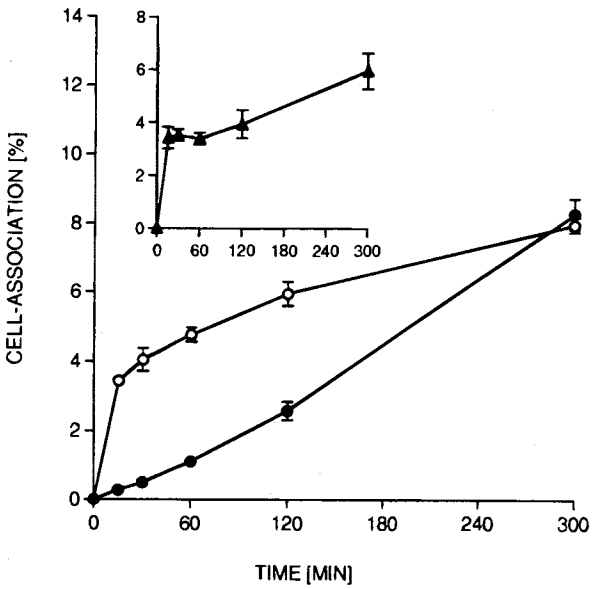

Fig. 1. Effect of time on the cellular association of PO and PS oligonucleotides (PO = filled circles, PS = open circles). Caco-2 monolayers were incubated with 0.4 μM oligonucleotide at 37°C and pH 7.2 in serum free uptake medium. Inset shows the time course for the cellular association of 5μM[^{3}H] ISIS3521 (filled triangles). Cell-associated oligonucleotide expressed as a percentage of the total amount applied to the monolayer, n = 5 or 6 ± standard deviation.

association was less than its end-labelled counterpart. In contrast, the [^{32}P]-labelled PO revealed an almost linear cellular association-time profile.

To assess the stability of the end-labelled oligonucleotides during exposure to the Caco-2 monolayer, apical samples were collected and analysed using 20% PAGE. Dephosphorylation of both 5′-end [^{32}P]-labelled PO and PS oligonucleotides was observed as early as the 30 min incubation and total cleavage of the label from these oligonucleotides was observed after 300 min. This was evident from the appearance of the free phosphate product (Figure 2). However, no other oligonucleotide degradation products were observed for either chemistry suggesting that oligonucleotides could remain intact in the culture conditions providing the label was not a substrate for phosphatases such as the internal tritium label. The availability of [^{3}H]-labelled oligonucleotide, ISIS3521, which was a gift from ISIS Pharmaceuticals Inc., was limited and thus, could only be used in some of the studies. The majority of the experiments were performed with end-labelled oligonucleotides but these experiments were restricted to a 15-minute incubation period during which these oligonucleotides were known to retain their label. Furthermore, 15 mins was all that was required for the completion of the initial rapid phase of oligonucleotide cell association, and is the focus of this study.

Influence of Temperature on Cellular Association

To assess the temperature-dependence of cell-association, PO and PS oligonucleotides were incubated at 6 different temperatures between 37 and 4°C (Fig. 3). Surprisingly, the cellular association of PO and PS oligonucleotides demonstrated differences in relation to temperature-dependence; PS cell-association was temperature-independent with a mean value of 4.2% (Fig. 3B) whereas that of the PO decreased from 0.2% at 37°C to 0.05% at 18°C and was then relatively constant at lower temperatures (0.05% at 4°C).

Cellular Association-concentration Profile and the Influence of Competitors

The concentration-dependence of the cellular association was determined by adding increasing amounts of unlabelled oligonucleotide to the incubation solutions containing a fixed amount of radiolabelled oligonucleotide (Fig. 4). At a total concentration of 0.4 μM the cellular association of PS oligonucleotide (3.0%) was an order of magnitude greater than that of the PO (0.2%). With increasing concentration of the unlabelled PS, competition with the radiolabelled component was observed as low as 2 μM (2.0%) which increased up to 20 μM (0.34%). This saturation of PS cell association was observed at both 37 and 4°C. Using the same concentration range, saturation for the PO oligonucleotide was not observed (0.2% at 20 μM); however, the percentage cellular association was reduced by 50% upon competition with 200μM unlabelled PO. Interestingly, ATP did not significantly compete for the cellular association of PS oligonucleotide and only a modest competition at 1000 μM was observed in the case of PO oligonucleotide (data not shown).

In a cross-competition study, the simultaneous incubation of 0.4 μM labelled PO oligonucleotide with 20 μM unlabelled PS resulted in a 50% decrease in the PO cellular association. Conversely 20 μM unlabelled PO had no effect on labelled PS oligonucleotide association indicating the greater affinity of the PS modification for the cell surface binding sites (Table I).

Effect of Oligonucleotide Length on Cellular Association

In order to study the influence of oligonucleotide size on cell association, it is necessary to remove sequence composition variations by maintaining a constant base ratio amongst the different oligomer lengths. In an attempt to produce "lengthmers" independent of sequence, we synthesised oligonucleotides to contain only the AGCT repeating motif. Using PS modified oligonucleotides only, an increase in oligonucleotide length led to a greater cellular association (data not shown). Cellular association of 1% was observed for the 8 mer and each subsequent addition of two AGCT motifs (8 bases) led to an increase of approximately 1.75% (data not shown). Interestingly, the amount of cellular association observed for the 24 mer PS oligonucleotide consisting of six AGCT repeat motifs (mean = 4.5%) was similar to that obtained with the 20 mer PS oligonucleotide antisense to the *tat* sequence in HIV-RNA (mean = 4.8%). The latter is the sequence used in the majority of the experiments reported in this study.

Influence of pH on Oligonucleotide Partition Coefficients and Cellular Association

The cellular association of PO and PS oligonucleotides was pH-dependent (Figures 5A and 5B). The effect of pH on both backbone types was comparable; in each case a decrease in pH from 7.2 to 5.0 resulted in a 2 to 3-fold increase (significantly significant at $p = 0.01$) in their cellular association (PO, 0.2 and 0.5%, PS, 4.7 and 11.3% at pH 7.2 and pH 5.0, respectively).

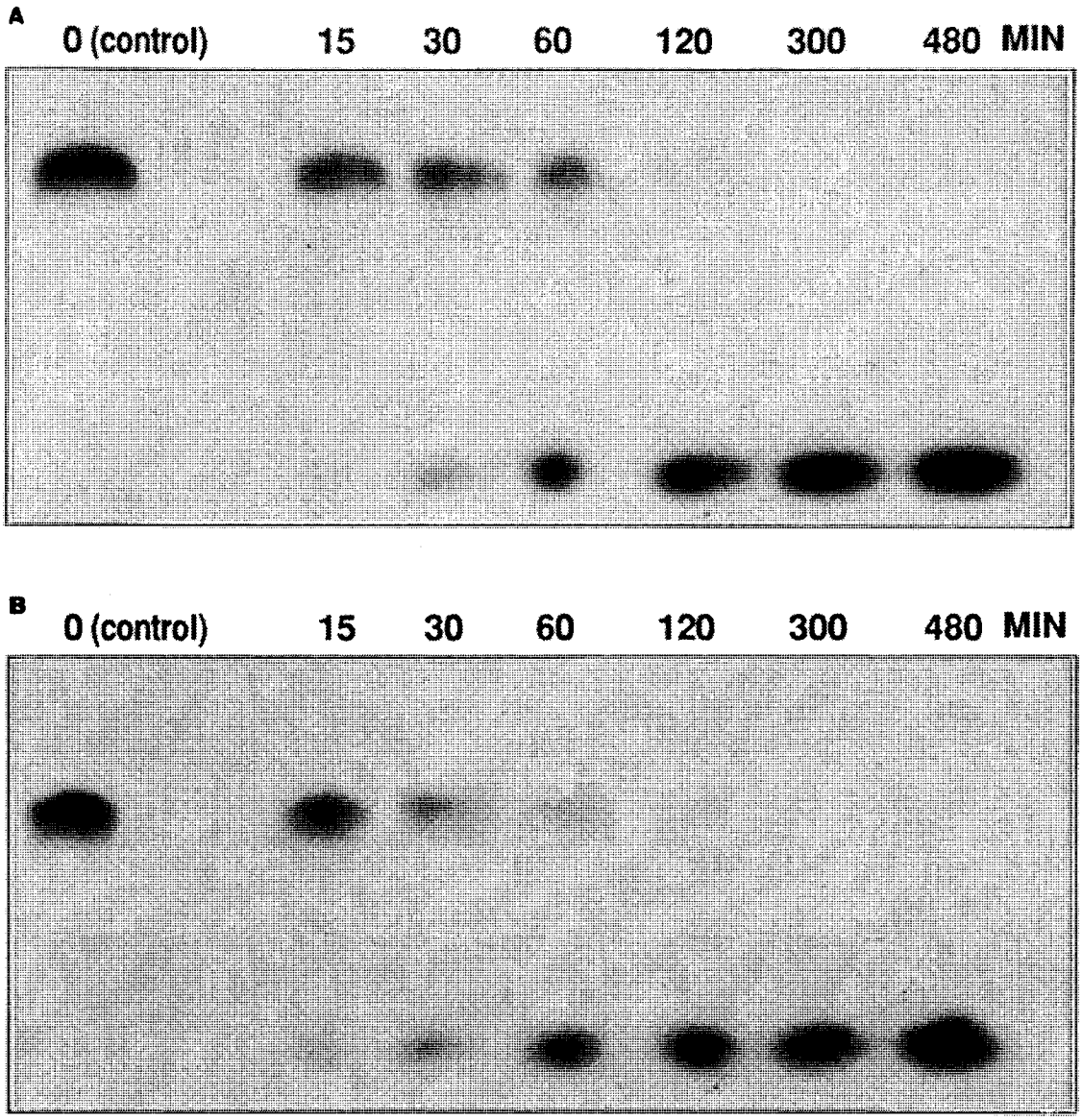

Fig. 2. Stability of 5′-[^{32}P]-labelled PO and PS oligonucleotides in Caco-2 monolayer apicals (PO, A; PS, B). The apical solutions of 0.4 μM oligonucleotide in serum-free uptake media were collected after 0 (Control), 15, 30, 60, 120, 300, 480 minutes and analyzed by 20% PAGE. The top band indicates the intact 20 mer oligonucleotide whereas the lower band represents 'free' radiolabelled phosphate that has been removed by dephosphorylating enzymes.

To assess whether the increased cellular association of oligonucleotides at low pH resulted from an increase in partition coefficient, octanol:aqueous buffer distribution coefficients were measured over this range (Figure 5C). For both backbone types no change in the distribution coefficient was observed between pH 8.0 and 5.0. However, the PO backbone exhibited higher distribution coefficients than PS at pH values of 4 and below (Figure 5C). Both the 20 mer PS sequences (i.e. *tat* and the ISIS sequence) exhibited similar distribution coefficients and showed no change over the entire pH range studied (data not shown).

Influence of the Ionic Environment on Cell Associated Oligonucleotide

To assess the effect of the ionic environment on cell-associated oligonucleotide, sodium chloride washes (post-incubation) were performed at a range of concentrations up to 1M NaCl (Table 1). These were able to remove up to 68% of the cell-associated PS oligonucleotide but were unable to remove any of the bound PO. The greatest amount of removal was observed with the highest ionic wash concentration (1M). Treatment with 1M NaCl for 5 mins was well tolerated by Caco-2 cells as monolayer viability and appearance was unaffected when assessed by trypan blue exclusion assay or when examined microscopically.

Assessment of Cell Surface Protein-binding

To estimate the protein-bound component of oligonucleotide cell-association, the Caco-2 monolayers were washed with a proteolytic enzyme, pronase (Fig. 6). For both backbone types, around 50% of the cell-associated oligonucleotide was pronase-

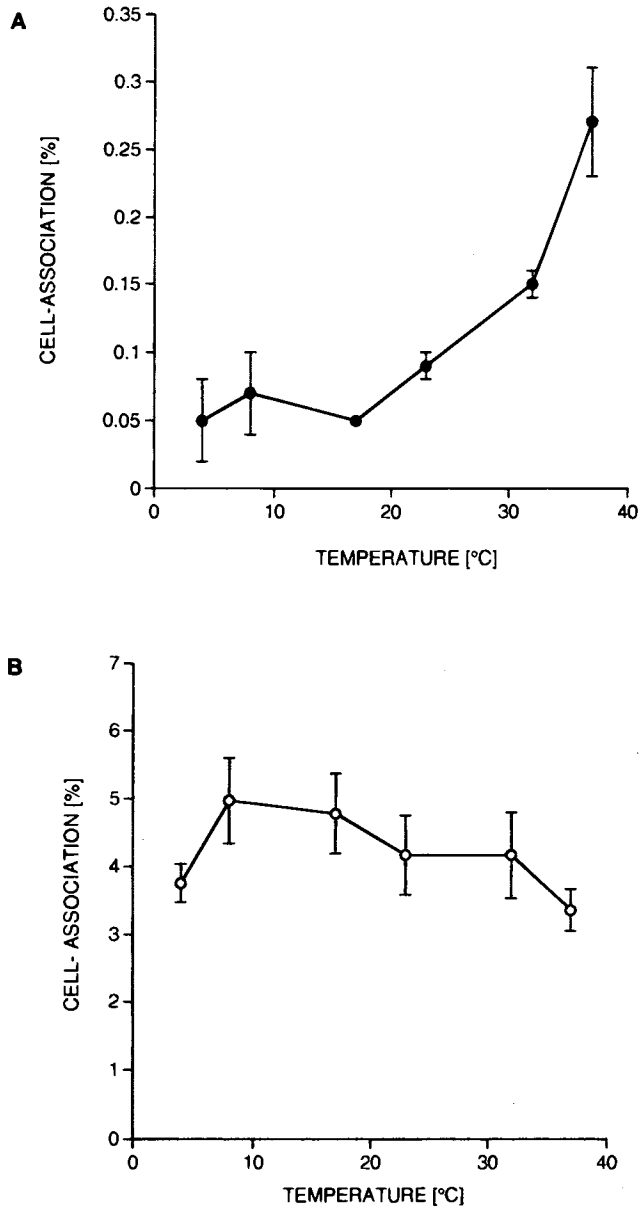

Fig. 3. Effect of temperature on the cellular association of PO and PS oligonucleotides (PO, A; PS, B). Caco-2 monolayers were incubated with 0.4 μM of oligonucleotide for 15 minutes at 4, 8, 17, 23, 32, 37°C and pH 7.2 in serum-free uptake medium. The cell-associated oligonucleotide was expressed as a percentage of the total amount applied to the monolayer, n = 6 ± standard deviation.

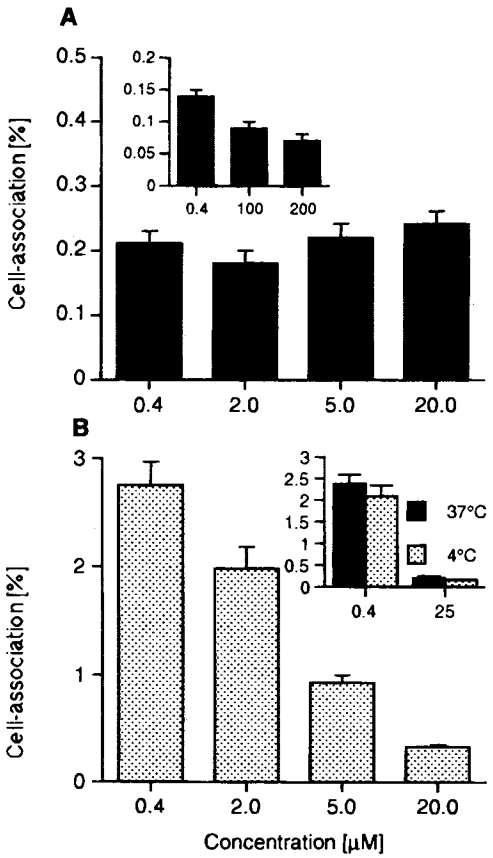

Fig. 4. Effect of concentration on the cellular association of PO and PS oligonucleotides (PO, A; PS, B). Caco-2 monolayers were incubated with 0.4, 2.0, 5.0, 20 μM oligonucleotide for 15 minutes at 37°C and pH 7.2 in serum-free uptake medium. Graph A inset; saturation of the cellular association of PO over an extended concentration range. Graph B inset; temperature-dependence of PS saturation. Cell-associated oligonucleotide expressed as percentage of the total amount applied to the monolayer, n = 5 or 6 ± standard deviation. For PO, statistically different cell association from the control value (0.4 μM) was only observed at 100 and 200 μM concentrations (p = 0.05). In contrast cell association of PS at 37°C was significantly different at all the concentrations shown (2–20 μM) (p = 0.01).

dependent at 37°C (Fig. 6A and C). However, at 4°C, a greater proportion of the cell-associated PO was pronase-sensitive (87%); whereas, the PS exhibited a similar profile to the 37°C results (50%). At pH 5.0, the pronase-sensitive component of PO cell-association at 37°C was increased 5-fold and that of PS increased 2-fold (Fig. 6B and D). At 4°C decreasing the pH had little effect on the pronase-sensitive component for either modification.

Clearly, the pronase-sensitive portion of the cell associated oligonucleotide is surface-bound, most probably to proteins, the remaining component, however, may have become internalized or even represent tight binding to either non-pronase sensitive (glyco)proteins, or even cell surface lipids [12–13] Treatment of Caco-2 cells with the metabolic inhibitors, sodium azide (10mM) and 2-deoxyglucose (50mM), prior to measuring cell association indicated that up to 50% of PO oligonucleotide association involved an energy-dependent process whereas cell association of PS oligonucleotide appeared to be an energy-independent process (Table I).

Comparison of Oligonucleotide Cell Association in Caco-2 with Other Epithelial Cell Lines

In an attempt to compare the magnitude of oligonucleotide cellular association observed in Caco-2 cells with other epithelial cell lines, we also examined PS and PO oligonucleotide association in confluent monolayers of Wish (amnion epithelium), T47D (Breast), T24 (bladder), Hep-G2 (liver), and A431 (vulva) cells. All the cell lines formed confluent monolayers

Table I. The Influence of Competitors and Metabolic Inhibitors on Cellular Association of Oligonucleotides. The Reduction in Cell Association of Labelled Control in the Presence of Competitors Is Expressed as % Inhibition in Cell Association, n = 6 ± s.d. Except Where Stated, Competitor Was Added Simultaneously with the Labelled Oligonucleotide to Cells

Competitor/Inhibitor		% Inhibition in Cell Association	
		PO	PS
[a]Self-competition with unlabelled oligomer, 20 μM		1 ± 4	88 ± 5
200 μM		50 ± 7	ND[e]
Cross-competition with unlabelled oligomer of opposite chemistry[b]		50 ± 5	−7 ± 8
NaCl[c]	300 mM	4 ± 9	31 ± 8
	500 mM	9 ± 7	49 ± 6
	1000 mM	4 ± 6	68 ± 7
Metabolic inhibition with 10mM sodium azide and 50mM 2-deoxyglucose[d]		33 ± 4	9 ± 9

[a] In the self competition, the corresponding unlabelled oligomer was added at the stated concentration simultaneously with the labelled oligonucleotide.

[b] In the cross-competition, the unlabelled oligonucleotide of identical sequence but of the opposite chemistry is added at the stated concentration to the labelled oligonucleotide.

[c] A NaCl wash (0.5 ml) at the stated salt concentration was used to replace one of the PBS-azide wash steps (see Methods).

[d] Metabolic inhibitors were pre-incubated with cells for 60 mins prior to cell association experiments.

[e] ND = Not determined.

on 24-well plates and were used after 7 days in culture. Generally, the level of oligonucleotide association in Caco-2 cells was similar to the epithelia derived from the breast, bladder and vulva, However, the greatest amount of cell-association was seen for Wish and Hep-G2 cells, the results for these being approximately double the amounts seen in the other four cell lines. Similar trends with both PO and PS oligonucleotides were seen (see Figure 7). Although these results are useful in providing a reasonable perspective on the relative binding potential of oligonucleotides to the different cells, owing to the different cell shape, size and gross morphology, accurate quantification is inherently difficult. Furthermore, the degree of differentiation in Caco-2 cells, which may also be different amongst the other cells investigated above, can strongly influence oligonucleotide cell association (Beck, G. F., Irwin, W. J., Nicklin, P. L., Akhtar, S., unpublished data). Thus comparisons between different cells even in the same study, let alone, other literature reports, is complex and difficult.

DISCUSSION

The mechanisms by which oligonucleotides enter cells have been reviewed by Akhtar and Juliano [14] and include the involvement of fluid-phase, adsorptive and "receptor" or specific binding protein-mediated endocytosis. Although cell uptake mechanisms vary with factors such as cell type (see also below), a general view is that oligonucleotide uptake by endocytosis, at least in cultured cells, appears to be poor and inefficient [15–17]. Thus, the recent finding that stable oligonucleotides are bioavailable following oral administration [5] is of some interest.

In an attempt to understand further the interactions of oligonucleotides with the gastro-intestinal barrier, we report on the cell association interactions of PO and PS oligonucleotides with human intestinal Caco-2 cells. These cells, which undergo spontaneous enterocytic differentiation in culture, appear to be useful in vitro models for such studies [8–10]. Indeed, their functional (e.g., polarity) and morphological (e.g., expression of brush border villi) properties are thought to resemble those of the human small intestinal cells [18].

Caco-2 cells are also known to express significant levels of alkaline phosphatase [19] which can potentially dephosphorylate 5′ end [^{32}P]-labelled oligonucleotides and thus prevent their use in oligonucleotide cell association studies. We, therefore, examined the stability of 5′-end [^{32}P]-radiolabelled PO and PS oligonucleotides in culture (Figure 2). Although genuine nuclease activity was not observed for at least 300 min in culture, rapid phosphorylation of both 5′ end-labelled PO and PS oligonucleotides led to subsequent experiments with end-labelled oligonucleotides being restricted to a 15 minute time period. However, for the longer-term studies (up to 300 min) we used an internally tritium-labelled oligonucleotide as this label is not a substrate for phosphatases.

The cellular association of PS oligonucleotides with Caco-2 cells appeared to be biphasic, with a rapid initial binding phase that was complete within 15 mins, followed by a more slower phase indicative of cellular internalization (Figure 1). This is consistent with the data from a parallel study using the pre-monocytic cell line, THP-1 [20] in which cellular association of PS oligonucleotides were typically complete with 10–15 min. In Caco-2 cells, cellular association of PO oligonucleotide was an order of magnitude lower than the PS chemistry. This reflected the greater cell binding affinity of PS compared to PO oligonucleotides and was consistently observed in the five other epithelial cell lines investigated (Figure 7). In epithelial cells from the breast, liver, amnion, bladder and vulva, the cell association of PS oligonucleotide was typically more than 10–20 fold higher than PO oligonucleotide. The greatest oligonucleotide binding, for both chemistries, was observed for cells derived from the liver and amnion. This result suggests that although cellular binding and uptake is cell type-dependent, the relative affinities for PO and PS appear to be similar between cells. A recent study suggests that this may be due to the varying amounts of oligonucleotide binding proteins present [20].

In Caco-2 cells, we found that the cellular association of PO oligonucleotides differed from the PS modification in several other respects. The cell association of PO oligonucleotide was temperature-dependent and this is consistent with previous studies implicating an active mechanism of binding and internalization for these oligonucleotides [21–22]. However, the cell association of PS oligonucleotide appeared to be temperature-independent. The fact that PS cell association was also unaffected by metabolic inhibition with sodium azide/ 2-deoxyglucose treatment (Table I), suggests that the major interactions during this initial phase of cell association are non-energy requiring mechanisms.

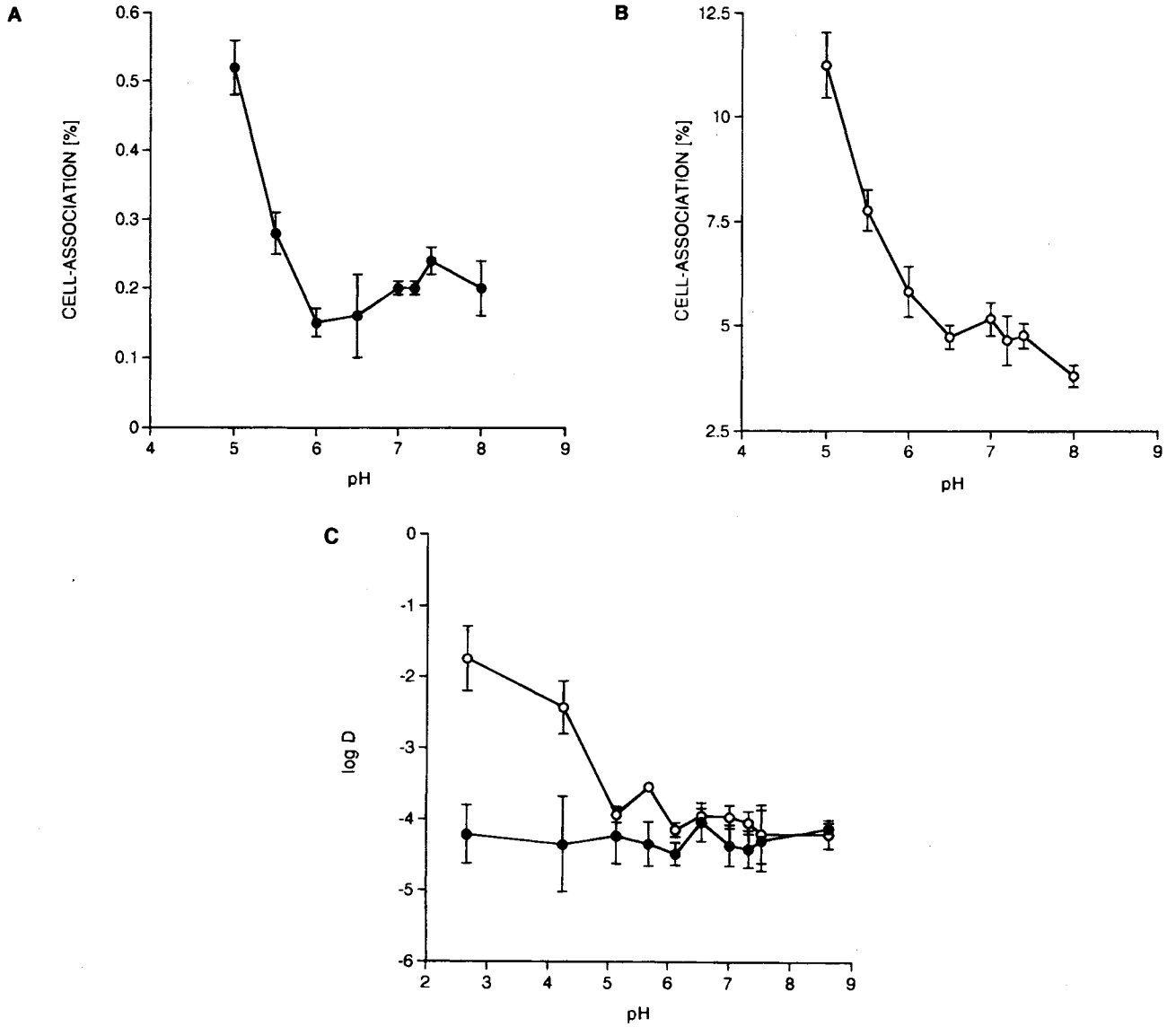

Fig. 5. Effect of pH on the cellular association and partition coefficients of oligonucleotides. For the effect of pH on cellular association of PO (A) and PS (B), Caco-2 monolayers were incubated with 0.4 μM of oligonucleotide for 15 minutes at 37°C and pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.2, 7.4, 8.0 in serum-free uptake media. Cell-associated oligonucleotide expressed as a percentage of the total amount applied to the monolayer, n = 6 ± standard deviation. For the effect of pH on n-octanol:aqueous buffer distribution coefficients for both oligonucleotides (C), distribution coefficients are expressed as log D, n = 3 ± standard deviation. Filled circles (PO) and open circles (PS).

The saturable and self-competitive nature of the cellular association observed for PS oligonucleotides is indicative of a specific cell surface-binding mechanism [16,21,23,24] which in theory could be mediated by binding proteins or even lipids. Indeed, studies with model lipid bilayers suggest that PS oligonucleotides bind more avidly to lipid components than PO oligonucleotides [12–13]. However, the cross-competition data also indicate that there is a degree of overlap in the binding sites that can be occupied by PO and PS oligonucleotides. Since PS and PO binding is reduced equally (approx. 50%) upon pronase washing, protein binding appears important with at least half of the binding occurring via cell surface proteins. The fact the NaCl washing can interfere, at least with PS oligonucleotides, indicates that cell surface binding to these sites involves ionic interactions. Interestingly, NaCl washes were unable to remove any of the cell-associated PO oligonucleotide and the reasons for this are unclear. It has been postulated that non-specific binding of DNA to ligands may be more sensitive to salt concentration than specific binding [25] thereby suggesting that protein binding of PO may be more specific than PS. Thus, it is likely that both oligonucleotide chemistries are able to interact with the Caco-2 cell-surface by ionic interactions, most probably, to proteins.

This theory is also re-enforced by the increased cellular association observed with a) increasing oligonucleotide length, which could be mediated by the increased charge and b) a decrease in pH, which, although not affecting the oligonucleotide would protonate the cell-surface proteins and hence make them more attractive to the negatively charged oligonucleotide.

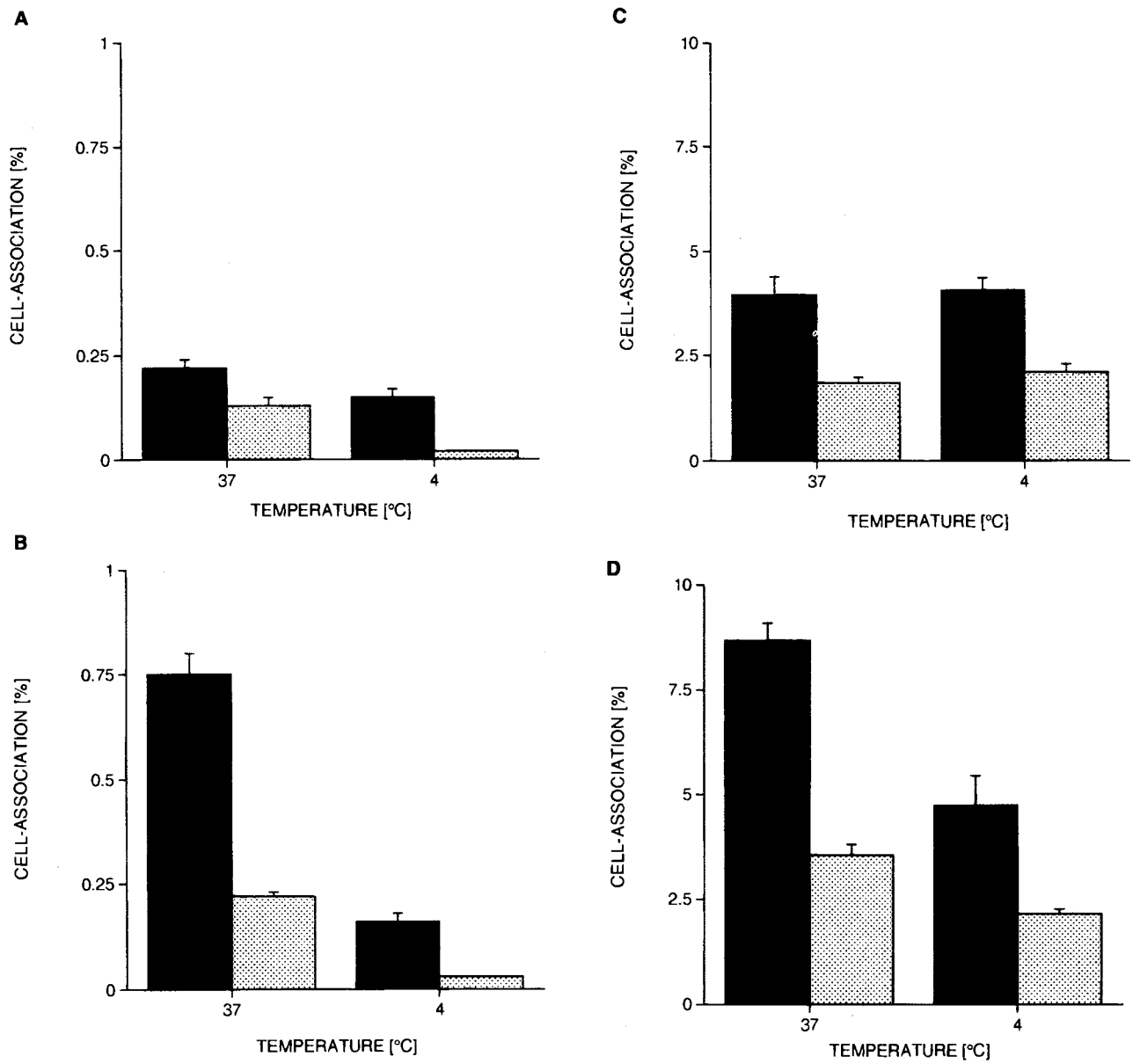

Fig. 6. Effect of Pronase washes on the cellular dissociation of PO and PS oligonucleotides (Control, filled; Pronase treated = shaded). Caco-2 monolayers were incubated with 0.4 μM of oligonucleotide for 15 minutes in serum-free uptake medium. This was done at 37 and 4°C, and pH 7.2 and 5.0 followed by 45 min incubation with 0.25% (w/v) pronase at 4°C (A & B, PO at pH 7.2 & 5.0; C & D, PS at pH 7.2 & 5.0 respectively). The cell-associated oligonucleotide was expressed as a percentage of the total amount applied to the monolayer, n = 6 ± standard deviation. In each case, pronase-treated samples yielded significantly different cell association values compared to the non-pronase-treated controls ($p = 0.05$).

This, however, is unlikely to be the whole story as both pronase and NaCl washes are unable to remove all the cell-associated oligonucleotide, even at 4°C when, theoretically, there should be no internalization. Possible protein sites for oligonucleotide binding include the ϵ-amino group of lysine, the guanidium group of arginine and the protonated imidazole of histidine [26]. Of these three sites, only the histidine with a pKa of 6.5 would be susceptible to protonation over the range 7.2 to 5.0. The others with pKa values greater than 10 (lysine, 10.2 and arginine, 12.0) would be fully protonated at the pH range under investigation. It may also, however, be protonation of the carboxylic acids causing a reduction in repulsion, rather than greater attraction, which leads to the increased cell-association over this pH range.

Our results on the influence of pH agree with the work of Goodarzi *et al.* [27], Kitajima *et al.* [28] and Hawley and Gibson [20] who also found that the binding of oligonucleotides to cell membrane proteins is enhanced under acidic conditions. Thus, it seems likely that binding, and presumably uptake, into intestinal cells *in vivo* may be greatest within the acidic regions of the gastro-intestinal tract; however, the interplay of other factors will also be important.

In this study we have shown that intestinal epithelial Caco-2 monolayers associate with PS oligomers in a high-

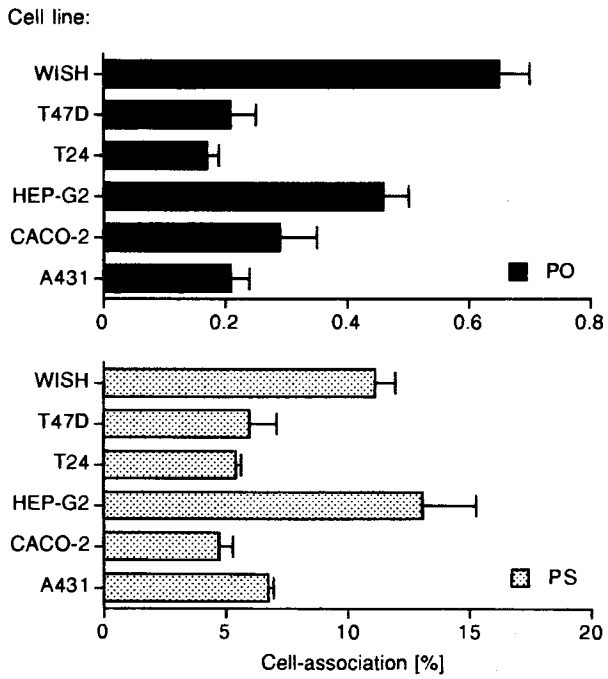

Fig. 7. Cellular association of PO and PS oligonucleotides to a range of human epithelial cell lines. Confluent monolayers of each cell line were incubated with 0.4 μM oligonucleotide in serum-free uptake medium for 15 minutes at 37°C and pH 7.2. Cell-associated oligonucleotide expressed as percentage of the total amount applied to the monolayer, n = 6 ± standard deviation.

affinity, capacity-limited fashion, and they exhibit binding to cell-surface components which can be increased by a reduction in pH. Furthermore, binding of PS consisted equally of pronase-sensitive and insensitive components whereas PO oligonucleotide binding was predominantly pronase-sensitive. These results in Caco-2 cells indicate that binding to surface proteins seems a major pathway for binding and internalization for both oligonucleotide chemistries and appear consistent with receptor (binding protein)-mediated endocytosis. Whether this binding protein-mediated entry of oligonucleotides can result in efficient transepithelial transport, however, requires further study. Caco-2 cells may serve as a useful model for such studies.

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