Calcium-dependent oligonucleotide antagonists specific for L-selectin

(SELEX/lymphocytes/endothelium/high endothelial venules/specificity)

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ABSTRACT   The selectins are calcium-dependent C-type lectins that recognize complex anionic carbohydrate ligands, initiating many cell–cell interactions in the vascular system. Selectin blockade shows therapeutic promise in a variety of inflammatory and postischemic pathologies. However, the available oligosaccharide ligand mimetics have low affinities and show cross-reaction among the three selectins, precluding efficient and specific blockade. The SELEX (systematic evolution of ligands by exponential enrichment) process uses combinatorial chemistry and *in vitro* selection to yield high affinity oligonucleotides with unexpected binding specificities. Nuclease-stabilized randomized oligonucleotides subjected to SELEX against recombinant L-selectin yielded calcium-dependent antagonists with ~10^5 higher affinity than the conventional oligosaccharide ligand sialyl Lewis^x. Most of the isolated ligands shared a common consensus sequence. Unlike sialyl Lewis^x, these antagonists show little binding to E- or P-selectin. Moreover, they show calcium-dependent binding to native L-selectin on peripheral blood lymphocytes and block L-selectin-dependent interactions with the natural ligands on high endothelial venules.

The selectin family of cell adhesion molecules plays critical roles in the initial events of leucocyte adhesion to vascular endothelium (1–11). Each of the three selectins (E, P, and L) has an amino-terminal C-type lectin domain that mediates calcium-dependent interactions with specific endogenous ligands. These high affinity ligands (1–8) include certain mucin-type glycoproteins carrying sialylated fucosylated polylactosaminoglycans (including sialyl Lewis^x), structural subsets of heparan sulfate glycosaminoglycans, some sulfated glycolipids, as well as certain peptide sequences with tyrosine sulfate residues (12–14). In addition, a variety of nonbiological macromolecules such as yeast phosphomannan and sulfated algal fucoidan can be recognized with high affinity (4, 5). The structural basis for the selective recognition of these apparently varied ligands by each of the three selectins is not yet elucidated. The suggestion that unusual sulfated variants of sialyl Lewis^x are primarily responsible for the high affinity and monospecificity of some natural ligands (15) has not been borne out in direct analyses (16).§ Regardless, the common feature of all ligands is that they present hydrophilic polyanionic surfaces bearing multiple carboxylate, sulfate, or phosphate groups, in the form of a clustered O-linked saccharides (4, 5), heparan sulfate glycosaminoglycans (17–19), micellar sulfated glycolipids (20, 21), or combined oligosaccharide/peptide epitopes, involving sialylated oligosaccharides and tyrosine sulfate residues (12–14).

Abundant information indicates that selectin-ligand interactions play critical roles in the earliest steps of tissue injury following hypoxemia, reperfusion, or inflammation (8, 22–25). Thus, small molecule sialyloligosaccharide inhibitors of the selectins have potential therapeutic value in many pathological conditions, including ischemia-reperfusion injury, acute inflammatory states, and chronic immune responses (23–25). Indeed, soluble selectin antagonists with binding constants in the micromolar range [e.g., sialyl Lewis^x (sLe^x) analogs] are capable of blunting tissue injury in several *in vivo* models (23–25). However, these antagonists must be used in large quantities and are very expensive to prepare. Furthermore, they show relatively little selectivity between the three selectins (1–5, 8), precluding the preferential blockade of a specific selectin in a particular pathological situation. On the other hand, the natural macromolecular high affinity ligands specific to each selectin are not available in the amounts needed for such studies, and their precise structural recognition motifs remain uncertain (1–5). Thus, almost all attempts to date to obtain soluble high affinity inhibitors specific to each selectin have failed. Recently, Martens *et al.* (26) used a phage display library to isolate short peptide sequences showing relative specificity for E-selectin and K_d values in the nanomolar range. However, binding was not calcium dependent, some cross-reactivity with the other selectins was seen, and micromolar concentrations were required for inhibiting E-selectin-dependent cell adhesion and rolling (26).

While a common feature of the natural selectin ligands is a complex polyanionic surface (4), polyanions *per se* are not high affinity selectin antagonists. We reasoned that defined oligonucleotide structure(s) might bind to the same epitopes as the natural ligands and have used systematic evolution of ligands by exponential enrichment (SELEX) technology (27–30) to isolate high affinity oligonucleotide ligands to L-selectin.

MATERIALS AND METHODS

Materials. The sources of most of the materials used are noted in the individual methods sections or in the figure legends. All other chemicals used were of reagent grade and/or the highest quality available.

SELEX for Isolation of L-Selectin Ligands. L-selectin receptor globulin (LS-Rg) was prepared as described (31),

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immobilized on Protein A-Sepharose 4 Fast Flow (PAS; Pharmacia) overnight at 4°C in binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$) adjusted to pH 8.0 followed by extensive washes with unmodified binding buffer. Coupling efficiency was >95% based on the LS-Rg content of wash fractions quantified by ELISA. Coupling densities ranged from 8 pmol/μl of drained beads in the initial round to 2.4 fmol/μl in the final rounds. Typically, 10 μl of beads, total reaction volumes of 100–200 μl, and a 10:1 molar ratio of oligonucleotide to protein were used. Oligonucleotide pools were precleared for 30 min at 4 or 22°C with 250 μl of PAS beads and unbound oligonucleotide mixed with LS-Rg-PAS beads for 90 min at 4 or 22°C in binding buffer. Backgrounds were determined by incubating an equal quantity of the same oligonucleotide with PAS beads under identical conditions. Both sets of beads were washed identically with binding buffer, pH 7.4, until wash fractions of the control beads contained ~0.05% or less of the input oligonucleotide. Bound oligonucleotide was eluted with binding buffer containing EDTA in place of divalent cations (rounds 1–3, 50 mM EDTA; rounds 4–14, 5 mM EDTA). EDTA-eluted oligonucleotides from the LS-Rg-PAS beads were precipitated, reverse transcribed, amplified, *in vitro* transcribed, and gel purified (27).

The starting pool of oligonucleotide molecules (40N7), which contained a 40-nucleotide randomized region, was prepared by PCR amplification and T7 oligonucleotide polymerase transcription from a synthetic DNA template: 5'-TCGGGCGAGTCGTCTG-40N-CCGCATCGTCCTCCC-3' (Operon). PCR primers were as follows: 5n7, 5'-TAATACG-ACTCACTATAGGGAGGACGATGCGG-3'; 3n7, 5'-TCG-GGCGAGTCGTCTG-3'. Evolved pools were cloned and sequenced by standard procedures.

Inhibition of Selectin Binding to Immobilized Sialyl Lewis^x or 3'SO$_3$-Lewis^x. The binding of recombinant soluble receptor-globulin fusion proteins including the lectin and epidermal growth factor domains of L-, E-, and P-selectins (31, 32) to immobilized polyacrylamide-sLe^x or 3'-HSO$_3$Le^x (Syntesome, Munchen, Germany) was studied in an ELISA assay similar to that previously described (32), except that the assay buffers contained 20 mM Hepes, pH 7.45, 125 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 1% protease-free BSA and that autoclaved reagents and sterile materials were used throughout. All three selectins were used at 1 nM and were precomplexed with an optimal secondary antibody dilution as described previously for L-selectin (32). The starting randomized mixture of sequences (40N7) was used as a negative control. Background values were determined in the presence of 5 mM EDTA.

Competition of Binding of L-Selectin to Metabolically Labeled GlyCAM-1. Labeling and purification of ^{35}S-labeled

lymph node-secreted L-selectin ligand GlyCAM-1 was carried out by minor modifications of prior methods (33, 34) involving sequential wheat germ agglutinin and L-selectin affinity columns. The binding of ~3000 cpm of [^{35}S]GlyCAM-1 to LS-Rg immobilized on Protein A-coated 12-well plates was studied in the buffer used for the other ELISA assays—details will be published elsewhere.§ The oligonucleotides in serial dilution were mixed with labeled GlyCAM-1 prior to addition to the wells. After washing, bound molecules were eluted with EDTA and counted. Nonspecific binding was determined by adding 5 mM EDTA during initial binding.

Dissociation Constants and IC$_{50}$ Values. Oligonucleotide-protein dissociation constants were measured by nitrocellulose filter partitioning (27) at 4 or 22°C in binding buffer, and calculated by least square fits using the graphics program KALEIDAGRAPH (Synergy Software, Reading, PA). IC$_{50}$ values for ELISA inhibition assays were derived by fitting the data iteratively to a nonlinear least squares equation (SigmaPlot, Jandel, San Rafael, CA) and are reported as mean ± SD.

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation from EDTA or citrate anticoagulated whole blood of normal volunteers.

Binding of Oligonucleotide Pools to PBMCs. PBMCs were washed twice in 10 ml of Dulbecco's PBS without divalent cations (GIBCO 141-90-029) and resuspended in SHMCK buffer (140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 20 mM Hepes, pH 7.35). Cells were counted, viability confirmed by trypan blue exclusion (>99%), resuspended at 2 × 10^7 cells/ml in SHMCK plus 1% BSA, and used immediately. Cells in 25 μl of SHMCK buffer were combined with equal volumes of serially diluted ^{32}P-labeled oligonucleotide in 0.65-ml Eppendorf tubes and incubated on ice for 30 min with occasional gentle vortexing; 40–45 μl of the reaction was carefully layered on 50 μl of a 1:1 mixture of dionyl/dibutyl phthalate in a 0.65-ml Eppendorf tube and centrifuged for 5 min at 16,000 × g at 4°C. Tubes were frozen in dry ice/ethanol; tips were amputated into scintillation vials and counted without fluor in a Beckman LS2000. Oligonucleotide binding was calculated from duplicate determinations, using the fraction of input counts bound, total oligonucleotide concentration, and number of cells.

RESULTS AND DISCUSSION

Isolation of High Affinity L-Selectin Ligands. The starting oligonucleotide pool for SELEX (40N7) was nuclease-stabilized by 2'-amino pyrimidine nucleotides (35) and randomized at 40 positions (potentially 4^{40}, but in practice 6 × 10^{14}
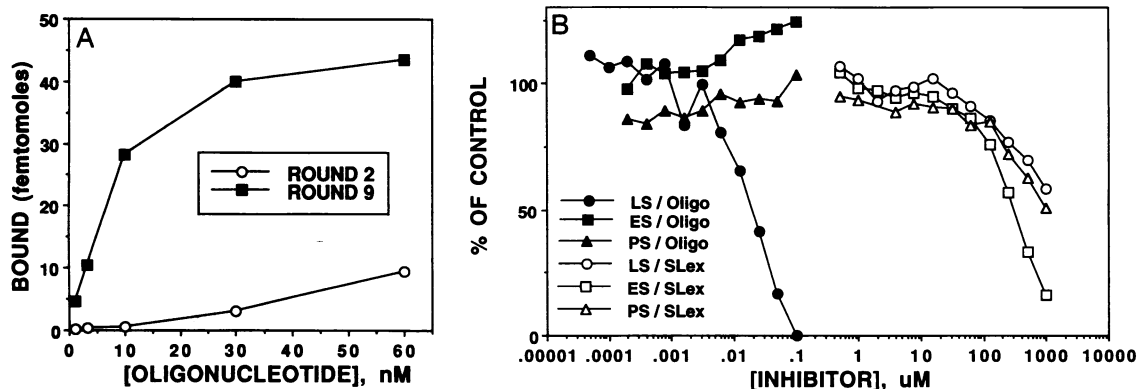

FIG. 1. High affinity binding of evolved oligonucleotide pools to human mononuclear cells and selective interaction with L-selectin. (*A*) Binding of second and ninth round SELEX oligonucleotide pools to human PBMCs was studied as described. (*B*) Inhibition of selectin binding to immobilized sialyl Lewis^x or 3'-SO$_3$-Lewis^x. Inhibition of the binding of recombinant soluble selectins to immobilized polyacrylamide-sLe^x or -HSO$_3$-Le^x by the enriched oligonucleotide pools indicated was studied as described. The starting randomized mixture of sequences (40N7) was used as a negative control.

Cell Biology: O'Connell et al.

Proc. Natl. Acad. Sci. USA 93 (1996)   5885

members). The apparent affinity of the starting pool for the target protein, a human L-selectin–Ig chimera (LS-Rg) (31) immobilized on Protein A-Sepharose beads, was 9 μM (data not shown). The strategy for selecting high affinity selectin antagonists was based on the premise that oligonucleotide ligands interacting with the lectin binding site would inhibit carbohydrate binding and be preferentially eluted by chelating the lectin domain's bound Ca^{2+} with low concentrations of EDTA (see Methods for details). The initial six rounds of SELEX were performed at 4°C. At the seventh round, the

SELEX was branched and carried in parallel at 4 and 22°C. The dissociation constants of the ninth (22°C) and 10th (4°C) round pools for purified L-selectin were 8 and 17 nM at 4°C, respectively (data not shown). Binding was specific for L-selectin in comparison with E- and P-selectin, divalent cation-dependent, and temperature-sensitive. After 14 rounds of SELEX (4°C), the evolved oligonucleotide pool's apparent dissociation constant was 0.3 nM, remained divalent cation-dependent, and exhibited a 300,000-fold improvement over the starting pool. Thus, the SELEX protocol enriched for a subset

A Ligand

| 6.79 | AUGUGUGAGUAGCUGAGCGCCCGAGUAUGAWACCUGACUA |
6.50	UAAUGUGUGAAUCAAGCAGUCUGAAUAGAUUAGACAAAAU
6.60	GGCAUUGUGUGAAUAGCUGAUCCCACAGGUAACAACAGCA
13.32	CGCGUAUGUGUGAAAGCGUGUGCACGGAGGCGUCUACAAU
14.21	UUGAGAUGUGUGAGUACAAGCUCAAAAUCCCGUUGGAGG
14.9	AAACCUUGAUGUGUGAUAGAGCAUCCCCCAGGCGACGUAC
14.25	UAGAGGUAGUAUGUGUGGGAGAUGAAAAUACUGUGGAAAG
6.28	GUAAAGAGAUCCUAAUGGCUCGCUAGAUGUGAUGUGAAAC
13.48	AAAGUUAUGAGUCCGUAUAUCAAGGUCGACAUGUGUGAAU
6.71	CACGAAAAACCCGAAUUGGGUCGCCCAUAAGGAUGUGUGA
14.12	UAACAACAAUCAAGGCGGGUUCACCGCCCCAGUAUGAGUA

CONSENSUS: AUGUGUGA
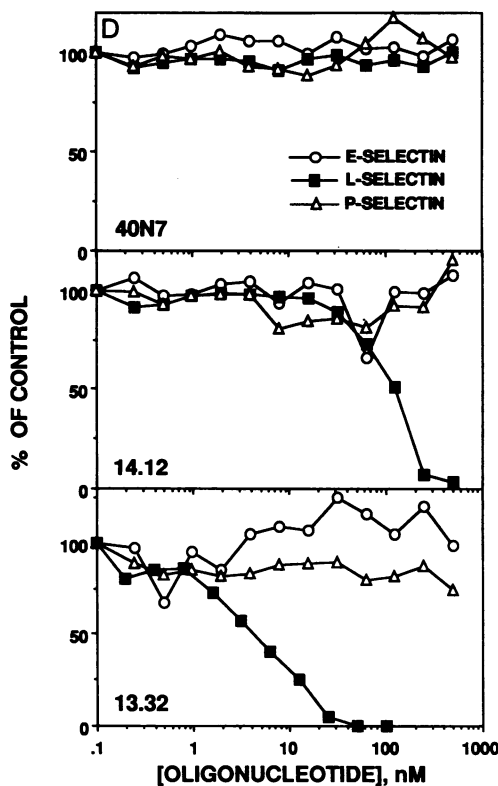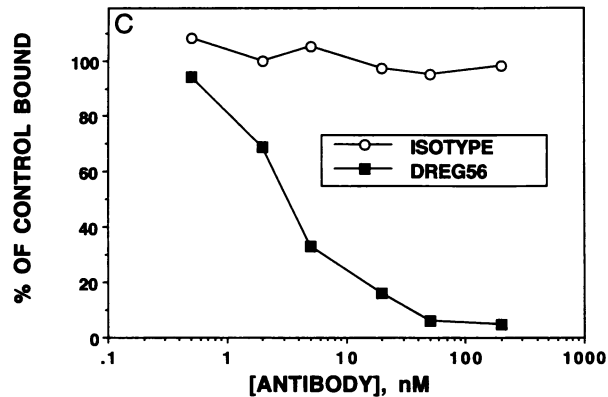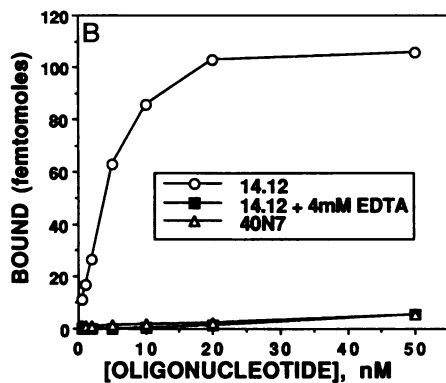
FIG. 2. Interaction of a cloned oligonucleotide ligand with L-selectin. (A) Primary structure of a family of SELEX ligands to L-selectin. An alignment of the evolved sequences for ligands isolated from rounds 6, 13 (22°C), and 14 (4°C) is shown. Ligand designations specify round and isolate number. The sequence of ligand 14.12 was observed in 69 clones; 35 of these contained the sequence AUGAGUG rather than AUGAGUA. (B) Binding of the cloned 14.12 oligonucleotide ligand to human peripheral blood mononuclear cells. The cell binding assays were carried out exactly as described in Fig. 1A. For determining divalent cation dependence, the buffer contained 4 mM EDTA in place of these cations. (C) Inhibition by anti-L selectin MAb (DREG56) of ligand 14.12 binding to human PBMCs. Assays were performed as described in Fig. 1A except that aliquots of a ^{32}P-labeled 14.12 oligonucleotide were combined with various concentrations of DREG56 (Endogen) or an isotype matched control antibody (anti-KLH, Becton Dickinson) prior to mixing with cells. Oligonucleotide bound at various antibody concentrations is expressed as the percent of that bound in their absence. (D) Inhibition of selectin binding to immobilized sialyl Lewis^x, by cloned oligonucleotides - comparison to the randomized pool 40N7. The ELISA assays were carried out as described in Fig. 1B.

of oligonucleotide sequences that likely bind with high affinity to the calcium-dependent C-type lectin domain of L-selectin (although divalent cation dependence of oligonucleotide structure cannot be ruled out).

The ninth round, but not the second round pool, showed high affinity saturable binding to human PBMCs, which are known to express L-selectin (Fig. 1*A*), indicating that ligands selected against the immobilized, purified protein are able to recognize native L-selectin in the context of a cell surface. In static ELISA assays (Fig. 1*B*), the 10th round pool inhibited the binding of purified L-selectin to immobilized sLe^x with IC$_{50}$s of 18 nM ($\pm$ 4.5) and 3'-SO$_3$-Le^x (6 nM $\pm$ 4) (n = 2, see *Methods* for details). Complete inhibition is seen at concentrations of 100 nM, and this concentration has no effect on the binding of E- and P-selectin receptor-globulin chimeras (31, 32) to the same ligands (Fig. 1*B*). The specific inhibition of L-selectin agrees with the above mentioned binding specificity. In contrast, sialyl Lewis^x, a well recognized natural oligosaccharide inhibitor of the the selectins, is ~10,000-fold less effective than the oligonucleotide pool and, unlike the latter, shows very limited selectivity among the three selectins (Fig. 1*B*). At a 100 nM concentration, the 10th round oligonucleotide pool also abolished the binding of LS-Rg to its natural ligands on the high endothelial venules of lymph nodes (data not shown). Taken together, these data confirm the specificity

of the inhibition of L-selectin and suggest that the oligonucleotide ligands are interacting with the calcium-dependent C-type lectin domain of L-selectin, which mediates physiological recognition in the vascular system.

Cloning and Sequencing of the Ligands Shows a Common Motif. Upon cloning, 75% of the ligands from the 14th (4°C) round pool and 35% from the 13th (22°C) pool had the same sequence, designated 14.12 (Fig. 2*A*). Ligand 14.12 gave a measured K_d of 0.2 and 3 nM to soluble L-selectin at 4 and 22°C, respectively. The affinity of 14.12 for L-selectin is 3,500–5,500-fold greater than for E- and P-selectin, and it showed the high affinity, saturable, divalent cation-dependent binding to human PBMCs found with the enriched oligonucleotide pool (Fig. 2*B*). The estimated number of oligonucleotide binding sites (0.5–1.3×10^5/cell) approximates the number of L-selectin molecules expected on these cells (36). Furthermore, binding was competitively inhibited by the anti-human L-selectin blocking monoclonal antibody DREG56 but not by an isotype matched control (Fig. 2*C*). Ligand 13.32 (Fig. 2*A*), isolated from the 22°C 13th round SELEX pool gave a K_d of 4 nM at 4°C and 3 nM at 22°C (i.e., it does not show the binding temperature sensitivity of 14.12). It also specifically binds to L-selectin on human PBMCs (data not shown). Both 13.32 and 14.12 inhibit L-selectin binding in the static ELISA assay with IC$_{50}$s of 9 ($\pm$8) and 37 nM ($\pm$29), respectively (n =
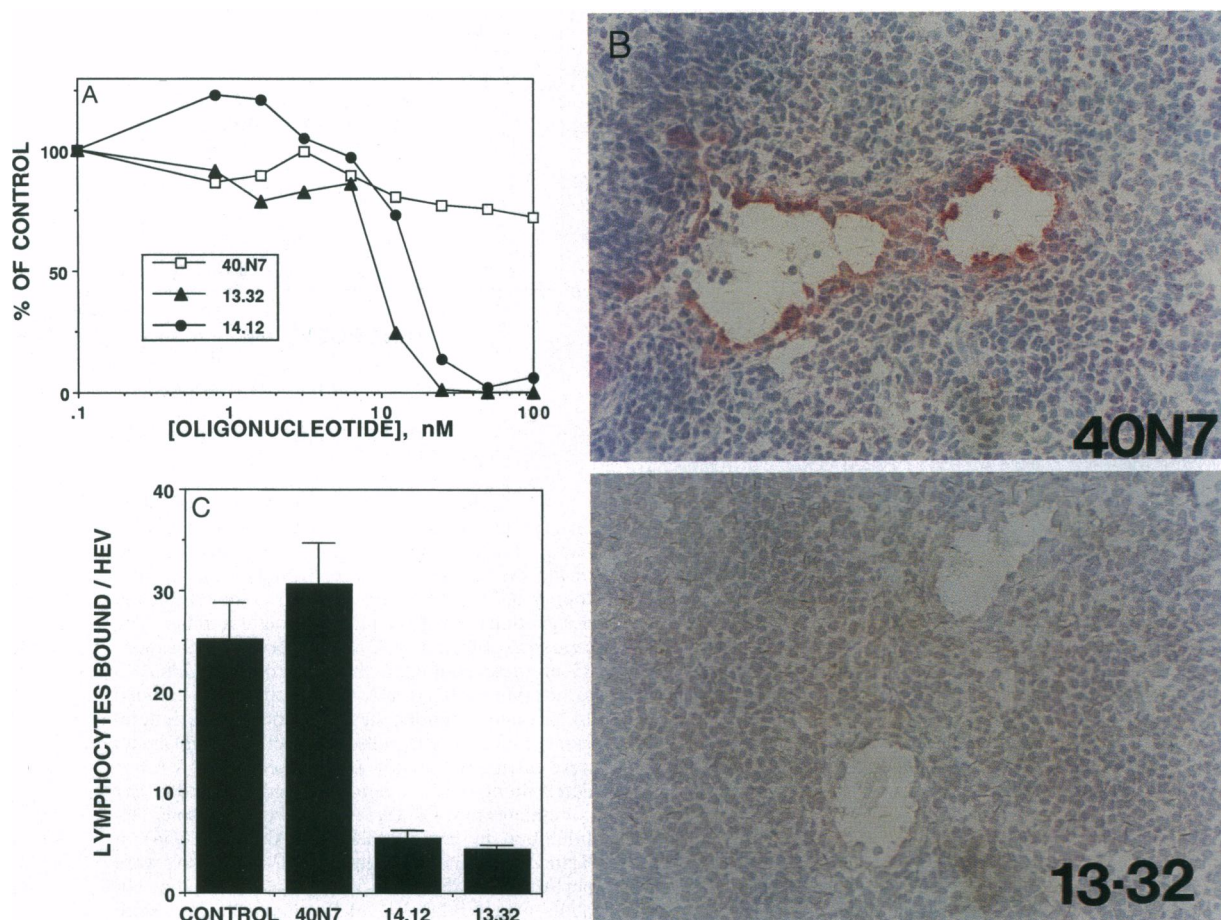

FIG. 3. Effects of cloned oligonucleotide ligands on the binding of L-selectin to natural ligands. (*A*) Competition of binding of L-selectin to metabolically labeled and purified GlyCAM-1 was carried out as described. Each point shown is [(average of duplicates) − (negative control)]/[(positive control) − (negative control)] expressed as a percentage. (*B*) Competition of staining of lymph node high endothelial venules with recombinant L-selectin. Staining was carried out as described previously (31). Initial LS-Rg binding was carried out in the presence or absence of the oligonucleotides at various concentrations. Examples are shown of 100 nM 40N7 (randomized pool, no effect) and of 13.32 (cloned ligand, complete inhibition). A control without oligonucleotide gave staining similar to that shown for 40N7 (data not shown). (*C*) Competition of binding of lymphocytes to the high endothelial venules of lymph nodes was studied by the Stamper–Woodruff assay (37), exactly as described previously (31). The representative experiment shown here shows the effects of adding the oligonucleotides at a final concentrations of 160 (40N7) or 80 nM (13.32 and 14.12).

4), while neither pool, at 500 nM, affects E- and P-selectin binding (Fig. 2D). Sequence alignment suggests that both ligands are members of a family with the consensus sequence, AUGUGUGA (Fig. 2A). Although it is probable that the consensus nucleotides are directly involved in binding L-selectin, preliminary boundary and truncation experiments demonstrate that additional sequence is required for high affinity binding (data not shown). Furthermore, high affinity ligands were observed in six additional, unrelated sequence families.

The High Affinity Ligands Block Binding of L-Selectin to Natural Ligands. All of the ELISA competition studies presented above involve the binding of L-selectin to artificial ligands (polyacrylamide-immobilized sLe^x or 3'-HSO$_3$-Le^x). To see if the cloned oligonucleotides can block binding to physiological ligands, we studied the interaction of L-selectin with the natural ligand GlyCAM-1, which was isolated from metabolically labeled high endothelial venules of mouse lymph nodes by affinity chromatography (33, 34). As shown in Fig. 3A, this interaction was inhibited with an IC$_{50}$ of 17 nM ($\pm$2, for oligonucleotide 14.12) and 9 nM ($\pm$0.4, for oligonucleotide 13.32), while the randomized mixture (40N7) was ineffective ($n = 2$). The cloned oligonucleotides (14.12 and 13.32) also specifically inhibited the interaction of LS-Rg (Fig. 3B) and of human PBMCs (Fig. 3C) with lymph node high endothelial venules at concentrations of 50–100 nM. The PBMC–high endothelial venule interaction study (Fig. 3C) uses the Stamper–Woodruff assay, with which L-selectin was originally discovered (37, 38). The IC$_{50}$ values in these assays are higher than the directly measured K_d values. This is likely because the assays cannot be made sensitive enough to measure IC$_{50}$ values in the picomolar range, i.e., the number of selectin molecules required for a readout gives a lectin concentration in the nM range, and the inhibitors must be present in excess.

Conclusions and Prospects. These studies demonstrate the first *in vitro* selection of nucleic acid ligands for cell surface receptors. Using this approach, we have prepared specific oligonucleotide ligands for L-selectin with IC$_{50}$ values that are at least 10,000–100,000-fold better than those of the oligosaccharide ligands (32) currently in clinical trials for the therapeutic modulation of selectin function, and of those suggested recently (15) as important in L-selectin binding to GlyCAM-1 (16). While the therapeutic use of nucleotides presents some challenges (28), technical progress in the modification and stabilization of oligonucleotides suggests ways in which potential problems may be circumvented. For example, nuclease susceptibility of oligonucleotides can be reduced by chemical alterations, such as the 2'-NH$_2$ modifications used here (35). However, since relatively short-lived infusions of oligosaccharides seem sufficient to attain significant therapeutic advantage in various pathological situations (22–25), it may not be necessary or even desirable to greatly enhance the stability of the oligonucleotides. The partial temperature sensitivity of binding affinity can be dealt with by carrying out the SELEX at physiological temperatures or by using alternative modifications. Finally, these studies provide high affinity, specific reagents for dissecting the roles of L-selectin in normal and pathological states. Since the published literature suggests that E- and P-selectin can be even more important in some pathological processes, SELEX experiments directed at obtaining unique high affinity nucleotide ligands for these receptors are also in progress. We anticipate that such molecules will allow the preferential blockade of a specific selectin in a particular physiological or pathological situation.

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