Abrogation of the Fc γ Receptor IIA-mediated Phagocytic Signal by Stem-Loop Syk Antisense Oligonucleotides

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The role of Syk kinase in Fc γ receptor (Fc γ R) IIA-mediated phagocytosis was examined with two forms of antisense oligodeoxynucleotides (ODNs) designed to hybridize to human Syk mRNA. Monocytes were incubated with linear and stem-loop antisense ODNs targeted to Syk mRNA. When complexed with cationic liposomes, stem-loop Syk antisense ODN with phosphorothioate modification exhibited stability in fetal bovine and human serum. The stem-loop Syk antisense ODN at a concentration of 0.2 μ M inhibited Fc γ RIIA-mediated phagocytosis by 90% and completely eliminated Syk mRNA and protein in monocytes, whereas scrambled-control ODNs had no effect. The Syk antisense ODNs did not change β -actin mRNA levels and Fc γ RII cell-surface expression. In addition, stem-loop Syk antisense ODN inhibited Fc γ RI and Fc γ RIIIA-mediated phagocytosis. These data indicate the efficacy of stem-loop Syk antisense ODN for targeting and degrading Syk mRNA and protein and the importance of Syk kinase in Fc γ receptor-mediated phagocytosis. Immunoblotting assay demonstrated that Fc γ RII tyrosine phosphorylation after Fc γ RII cross-linking did not change in the absence of Syk protein. These results indicate that Syk kinase is required for Fc γ RIIA-mediated phagocytic signaling and that Fc γ RII cross-linking leads to tyrosine phosphorylation of Fc γ RII independent of Syk kinase.

INTRODUCTION

Receptors for the Fc portion of immunoglobulin G (IgG)¹ are expressed on many cells of hematopoietic lineage (Ravetch and Kinet, 1991; van de Winkel and Anderson, 1991; McKenzie and Schreiber, 1994; Indik *et al.*, 1995b). These receptors are important in host defense and ingestion of IgG-coated cells. Three classes of Fc γ receptors have been described: Fc γ RI, Fc γ RII, and Fc γ RIII, distinguished by IgG subclass affinity, cellular distribution, and reactivity with monoclonal antibodies (Ravetch and Kinet, 1991;

McKenzie and Schreiber, 1994; Indik *et al.*, 1995b). The 40-kDa glycoprotein Fc γ RII has low affinity for monomeric ligand and interacts only with polymeric IgG (Stuart *et al.*, 1987; Hibbs *et al.*, 1988). Fc γ RII is expressed on a wide variety of cells and frequently represents the sole Fc γ receptor class expressed on hematopoietic cells (e.g., B lymphocytes, eosinophils, and platelets). Human Fc γ RII is encoded by three genes: Fc γ RIIA, IIB, and IIC (Brooks *et al.*, 1989; Cassel *et al.*, 1993).

The cytoplasmic domain of Fc γ RIIA contains two YxxL sequences, similar to those found in other Ig gene family receptors. The YxxL sequences in the cy-

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¹ Abbreviations used: EA, IgG-sensitized red blood cells; Fc γ R, Fc γ receptor; IgG, immunoglobulin G; ODN, oligodeoxynucleotides; PI, phagocytic index; RBCs, red blood cells; RT-PCR, reverse-transcribed polymerase chain reaction.

toplasmic domain of Fc γ RIIA account for its ability to mediate phagocytosis and tyrosine phosphorylation (Indik *et al.*, 1994b; Mitchell *et al.*, 1994). Two other isoforms of Fc γ RII, Fc γ RIIB1 and Fc γ RIIB2, contain only a single YxxL sequence and do not mediate phagocytosis (Indik *et al.*, 1994b).

Fc γ RII cross-linking results in the tyrosine phosphorylation of multiple proteins, including the receptor itself (Huang *et al.*, 1992; Scholl *et al.*, 1992; Rankin *et al.*, 1993; Ghazizadeh and Fleit, 1994), and induces rapid hydrolysis of phosphatidylinositol 4,5-diphosphate by PLC- γ 1 (Liao *et al.*, 1992). Pretreatment of cells with tyrosine kinase inhibitors substantially reduces Fc γ RII-mediated effector functions, including calcium mobilization, tyrosine phosphorylation, and phagocytosis (Liao *et al.*, 1992; Scholl *et al.*, 1992; Rankin *et al.*, 1993; Ghazizadeh and Fleit, 1994; Mitchell *et al.*, 1994).

The protein tyrosine kinase Syk is expressed in several hematopoietic cells, including monocytes/macrophages and platelets (Taniguchi *et al.*, 1991; Law *et al.*, 1994; Yagi *et al.*, 1994). The induction of Syk phosphorylation by cross-linking of Fc γ RII further suggests that Syk plays an important role in phagocytic signaling through Fc γ RIIA (Agarwal *et al.*, 1993; Kiener *et al.*, 1993; Greenberg *et al.*, 1994; Hunter *et al.*, 1994; Pan *et al.*, 1994; Ghazizadeh *et al.*, 1995; Indik *et al.*, 1995a). In a model system Syk kinase enhances the phagocytic signal induced by Fc γ RIIA and increases the number of cells able to mediate phagocytosis (Indik *et al.*, 1995a), but the critical position of Syk kinase in the signal cascade mediated by Fc γ RIIA is still unclear.

Antisense oligodeoxynucleotides (ODNs) designed to hybridize to specific mRNA sequences have been used to inhibit the expression of a number of cellular and viral genes (Chiang *et al.*, 1991; Milligan *et al.*, 1993; Stein and Cheng, 1993; Wagner *et al.*, 1993, 1994). Binding of antisense ODNs to their complementary sequences leads to either degradation of target mRNA by the RNaseH activity or blockage of the ribosomal translation of target mRNA (Milligan *et al.*, 1993; Stein and Cheng, 1993; Wagner, 1994). The development of effective antisense techniques allows investigators to address questions regarding gene function (Chiang *et al.*, 1991; Milligan *et al.*, 1993; Stein and Cheng, 1993; Wagner *et al.*, 1993, 1994). We have designed three linear ODNs and a stem-loop antisense ODN for human Syk mRNA. The stem-loop antisense ODN was designed to hybridize to three different sites of human Syk mRNA. These Syk antisense ODNs were used to investigate the role of Syk kinase in the Fc γ RIIA-mediated phagocytic signal in cultured monocytes. We report that the stem-loop antisense ODN structure may provide an improved approach to target mRNA and protein. By using this approach, we demonstrate that the protein tyrosine kinase Syk is required in Fc γ RIIA-mediated phagocytic signaling and examine

the requirement for Syk kinase in Fc γ receptor tyrosine phosphorylation.

MATERIALS AND METHODS

Construction of Antisense ODNs

Antisense and scrambled-control ODNs were modified to be protected from nuclease digestion. One phosphodiester backbone at the 5-prime terminus and two at the 3-prime terminus were modified with phosphorothioates. Prediction of secondary structures of Syk mRNA (Law *et al.*, 1994) and ODNs was performed with the MacDNASIS program (Hitachi Software, San Bruno, CA). Three linear Syk antisense ODNs were designed. Target-I linear Syk antisense ODN (5'-CTGTCAGCCATGCCG-3', nucleotides 159–173, according to the nucleotide numbering of Law *et al.*, 1994) targets the area surrounding the translation initiation codon of Syk mRNA. Target-II (5'-GCTTCTTGAGGAG-3') and target-III (5'-TGTCTTGCTTTGTC-3') linear Syk antisense ODNs are complementary to nucleotides 451–463 and nucleotides 802–816, respectively. Stem-loop Syk antisense ODN is a 57-mer-containing sequence complementary to these three target sites (Figure 1). The stem-loop Syk antisense ODN forms a stem and loop structure by itself (Figure 2). The stem domain, seven nucleotides in length, is formed by complementary 5- and 3-prime terminal sequences with nucleotide contents of only Gs and Cs. The loop domain consists of three antisense sequences complementary to targets I, II, and III. Three different antisense sequences were tandemly joined in 5' to 3' order for targets I, III, and II to contain minimum intramolecular secondary structure in the loop domain (Figure 2). The sequence of the stem-loop Syk antisense ODN is 5'-GGGGGGGCTGTCAGCCATGCCGTGCTTGTCTTTGTCGCTTCTTGAGGAGCCCCCCC-3'. The linear scrambled-control ODN is a 15 mer with the sequence 5'-CCAAGATGATTCCAG-3', and the stem-loop scrambled-control ODN is a 57 mer with 7 Gs and 7 Cs within the stem domain and a scrambled sequence 5'-ATGGAATCATCTTGGGCATTTCGTCTCTCAAAGAAGAATATG-3' within the loop domain. The linear and stem-loop control ODNs were also modified at both the 5-prime and 3-prime termini by phosphorothioates. All ODNs were synthesized by the Nucleic Acid Facility, Department of Chemistry, University of Pennsylvania Cancer Center (Philadelphia, PA).

Stability of Linear and Stem-Loop Syk Antisense ODNs

One microgram each of the nonspecific control-phosphodiester ODN and the specific target-I linear and stem-loop Syk antisense ODNs in 50 μ l of phosphate-buffered saline (PBS) were incubated with 2 μ g (1 μ l) of Lipofectamine (Life Technologies, Gaithersburg, MD). The ODN-liposome complexes were allowed to form at room temperature for 45 min. An equal volume of PBS containing non-heat-inactivated fetal calf serum (FCS; Intergen, Purchase, NY) or human serum from a healthy individual was added to each ODN-liposome mixture to a final concentration of 10%, 33% FCS, or 50% human serum and incubated at 37°C for 16 h. ODNs (1 μ g) without Lipofectamine were also incubated in 100 μ l of PBS with a final concentration of 10%, 33% FCS, or 50% human serum. After phenol and chloroform extraction, the ODNs were run on a 12% polyacrylamide gel, and ODNs were visualized by ethidium bromide staining.

Monocyte Isolation and Culture

Peripheral blood mononuclear cells from healthy individuals were isolated as previously described (Darby *et al.*, 1994). Briefly, the heparinized blood was centrifuged on Ficoll-Hypaque (Lymphocyte Separation Medium; Organon Teknica, Durham, NC), and interface cells were washed twice in PBS. Mononuclear cells were resuspended in complete medium containing RPMI-1640 (Life Technol-

ogies) with 10% heat-inactivated FCS and 2 mM L-glutamine. Cells were allowed to adhere at 37°C onto tissue-culture flasks precoated with FCS. After 45–90 min, nonadherent cells were removed by extensive washing in Hanks' balanced salt solution. Cells were harvested by vigorous agitation. Monocytes were routinely >98% viable as judged by trypan blue exclusion. Isolated monocytes were maintained in RPMI-1640 supplemented with L-glutamine (2 mM) and 10% heat-inactivated FCS at 37°C in 5% CO₂.

ODN Treatment of Cells

Monocytes (1×10^5 or 2.5×10^5) were seeded in each well of a 24-well plate (Becton Dickinson Labware; Falcon, Lincoln Park, NJ). ODN-liposome complexes were added twice, once on day 0 and once on day 1. Each time 2 μg of Lipofectamine and 1 μg of phosphorothioate ODNs (0.5 μM for the linear ODNs and 0.1 μM for the stem-loop ODNs) were allowed to form complexes in 100 μl RPMI-1640. The ODN-liposome complexes were added to each well containing monocytes in 0.2 ml of the culture medium without FCS. Transfection was performed at 37°C for 4 h. After transfection the culture medium was adjusted to 10% FCS in a final volume of 1 ml. The next day the same volume of ODN-liposome complexes (100 μl) was added again, and the monocytes were incubated at 37°C one additional day before assay. Monocytes cultured with the ODNs were >98% viable as judged by trypan blue exclusion.

Preparation of IgG-sensitized Red Blood Cells (EA)

Sheep red blood cells (RBCs)/ml (1×10^9 ; Rockland, Gilbertsville, PA) were sensitized with an equal volume of the highest subagglutinating concentration of rabbit anti-sheep RBC antibody (Cappel Laboratories, West Chester, PA) at 37°C for 30 min. The IgG-sensitized RBCs were washed twice and resuspended in PBS to a final concentration of 1×10^9 RBCs/ml, as described previously (Schreiber *et al.*, 1975).

Phagocytosis of EA

Monocytes treated with antisense ODNs were incubated at 37°C for 30 min with EA at a ratio of 100:1 (EA to monocytes). Cells were exposed briefly to hypotonic PBS to remove adherent EA. The cells were then stained with Wright-Giemsa, and phagocytosed RBCs were microscopically ($\times 1000$) scored. One hundred monocytes were chosen in a random manner, and internalized EA was expressed as a phagocytic index (PI; the number of ingested RBCs per 100 monocytes).

Flow Cytometry Analysis

Monocytes were incubated with anti-FcγRI (32.2) (Indik *et al.*, 1994b), anti-FcγRII (IV.3) (Indik *et al.*, 1994b), or anti-FcγRIII (3G8) monoclonal antibody (mAb) (Park *et al.*, 1993a,b) at 4°C for 30 min, washed twice with calcium and magnesium-free PBS containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide, and labeled with FITC-conjugated goat anti-mouse F(ab')₂ IgG (Tago, Burlingame, CA) at 4°C for 30 min. Cells were then washed and fixed with 1% paraformaldehyde. Fluorescence was measured on a FACStar (Becton Dickinson, Mountain View, CA), and mean fluorescence intensity data and contour plots were generated by Consort 30 software. For all samples 10,000 events were recorded on a logarithmic fluorescence scale.

Reverse-transcribed Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from monocytes (1×10^5 or 2.5×10^5 cells/ml) treated with scrambled-control or Syk-antisense ODNs. cDNA was synthesized from total RNA with random hexanucleotide primers (Boehringer Mannheim, Indianapolis, IN). PCR was performed

with synthesized cDNA as templates with two primers: Syk-H primer, 5'-GGTGTGTGCCCTCCGGCC-3' corresponding to nucleotides 122–139 of Syk mRNA (Law *et al.*, 1994), and Syk-M primer, 5'-CTGCAGGTTCATGT-3' (nucleotides 550–564). Human β-actin primer (Stratagene, La Jolla, CA) was used as a control. The PCR products of Syk were further analyzed by Southern hybridization.

Southern Hybridization

RT-PCR products were electrophoresed on a 1.5% agarose gel. DNA was transferred onto a nylon membrane (New England Nuclear Research Products, Boston, MA) for 5 h in 0.4N NaOH transfer solution. The membrane was hybridized with a biotinylated internal probe (Syk-pS, 5'-GGGAGTGGTAGTGCCAGAGG-3', nucleotides 408–427) in 6× SSPE and 50% formamide. After the membrane was washed in 0.1× SSC at 50°C, the hybridized bands were visualized with chemiluminescent detection reagent (ProtoGene Nucleic Acid Detection System; Life Technologies).

Antibody Stimulation, Immunoprecipitation, and Immunoblotting

Antibody stimulation, immunoprecipitation, and immunoblotting were performed as previously described (Darby *et al.*, 1994). Monocytes (1×10^5 cells/ml) treated with 0.2 μM of scrambled-control or Syk-antisense ODN were incubated with 50 μg/ml of anti-FcγRII mAb (IV.3) at 0°C for 30 min and centrifuged. The supernatant was aspirated, and the cells were incubated with 0.5 ml of goat anti-mouse F(ab')₂ IgG (50 μg/ml) (Organon Teknika) at 37°C for 4 min. After receptor cross-linking the cells were centrifuged, washed in ice-cold PBS, and lysed in 0.5 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM Tris-HCl, and 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) containing the following protease inhibitors: 1 mM sodium orthovanadate (Na₃VO₄), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, and 50 μg/ml of leupeptin. After a 40-min incubation at 0°C, the lysates were centrifuged at 4°C. Supernatants were harvested, and cell lysates were immunoprecipitated with the saturating amount of p72^{syk} antibody (Ab; kindly provided by Andrew C. Chan, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO) or anti-FcγRII mAb (IV.3) at 0°C for 2 h. Immune complexes were bound to 35 μl of washed *Staphylococcus aureus* cells (Pansorbin; Calbiochem, La Jolla, CA). Pellets were washed three times in lysis buffer, and adsorbed proteins were eluted into 20 μl of reducing-sample buffer. Proteins were resolved on SDS-PAGE and electrophoretically transferred to nitrocellulose. Immunoblots were probed with murine anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). After the membrane was stripped (55°C for 30 min in 2% SDS plus 0.7% 2-ME), immunoblots were prepared with anti-FcγRII mAb II1A5 (kindly provided by Jurgen Frey, Bielefeld, Germany) or p72^{syk} Ab (Upstate Biotechnology). Blots were developed with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA). Bound proteins were visualized with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Immunoblotting of p72^{syk}

Monocytes (1×10^5 cells/ml) treated with scrambled-control or Syk-antisense ODN were washed in ice-cold PBS and lysed as described above. Cell lysates corresponding to 2×10^5 cells were separated by SDS-PAGE, and immunoblots were performed with p72^{syk} Ab (Upstate Biotechnology). Blots were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and visualized with ECL.

RESULTS

Stability of Syk Antisense ODNs

The efficacy of antisense ODNs for ablating gene expression has not been unequivocal due, in part, to the sensitivity of the ODNs to digestion by nucleases, the difficulty with delivery into cells, and the inaccessibility of target sequences. To address some of these problems, we devised a novel antisense ODN structure with additional phosphorothioate modifications at both the 5-prime and 3-prime termini. The antisense ODN was designed to form a stem and loop structure (Figure 2). The stem domain contains only Gs and Cs for stable base pairing between the 5-prime and 3-prime termini and protects the loop domain. The loop domain consists of three antisense sequences complementary to different target sequences of Syk mRNA (Figure 1). The loop domain was designed to have minimal intramolecular secondary structure, allowing no more than three contiguous base pairs (bp) to be formed.

The stability of the stem-loop Syk antisense ODN was examined by incubation with FCS or human serum that was not heat inactivated. Control phosphodiester and antisense phosphorothioate ODNs were used either alone or complexed with cationic liposomes (Lipofectamine) for incubation with FCS or human serum. Whereas control phosphodiester ODNs were primarily degraded in FCS or human serum with or without Lipofectamine, target-I linear and stem-loop Syk antisense ODNs modified with phosphorothioates showed partial resistance to nuclease activity in FCS or human serum (Figure 3A). When complexed

with liposomes, linear and stem-loop antisense ODNs with phosphorothioate modifications at both the 5-prime and 3-prime termini were even more stable in serum (Figure 3B). Furthermore, the stem-loop Syk antisense ODN with phosphorothioate modifications exhibited further stability in both FCS and human serum, as indicated by the lack of degradation after 16 h incubation at 37°C (compare lanes 4, 8, and 12 in Figure 3B).

FcγRII Expression and Phagocytosis in Cultured Monocytes

Human monocytes express all three classes of Fcγ receptors when cultured at a cell density 1×10^6 cells/ml. However, when monocytes were cultured at 1×10^5 cells/ml for 2 d, the cells were found to express only FcγRII but not FcγRI or FcγRIII (Figure 4). FcγRI and FcγRIII are also not expressed on monocytes (1×10^5 cells/ml) cultured for 2 d with 200 units/ml of human recombinant macrophage colony-stimulating factor (rM-CSF). However, when cultured monocytes expressing all three classes of Fcγ receptors (1×10^6 cells/ml for 2 d) were further cultured at the lower cell density of 1×10^5 cells/ml for two more days in fresh medium, FcγRI and FcγRIII expression decreased, whereas FcγRII expression was unchanged. The mechanism for this observation is uncertain. Expression of FcγRI and FcγRIII may be influenced by a concentration of cytokines released by monocytes at a high cell density.

Monocytes cultured at 1×10^5 cells/ml were examined for Fcγ receptor-mediated phagocytosis. When

Figure 1. Selection of a target sequence for the stem-loop Syk antisense ODN. The cDNA sequence of human Syk mRNA is represented by the dotted rectangle (Law *et al.*, 1994). The entire Syk-mRNA sequence was scanned three times with an RNA secondary-structure prediction program to find sequences with minimum secondary structure. Each scanning was performed 33 bases apart in a 99-nucleotide frame (denoted as frames A, B, and C; top panel) sequentially. Putative secondary structures in the area of Syk mRNA containing the target-III sequence are shown in the three staggered frames of 99 nucleotides each (frames A, B, and C; bottom panel). Thus, the given sequence was scanned for putative secondary structure three times in three different frames. The most open sequence 5'-GACAAAGACAAGACA-3' (circled nucleotides in bottom panel) in three staggered scanings was chosen as a target-III sequence. Targets I and II were chosen in the same manner. The three target sites for the three linear and the

stem-loop Syk antisense ODN are shown below the Syk-mRNA sequence line as three short solid lines (denoted I, II, and III; top panel). Target sites I, II, and III correspond to nucleotides 159–173 (the area surrounding the translation initiation codon), 451–463, and 802–816, respectively.

Figure 2. Secondary structure of the stem-loop Syk antisense ODN. The stem domain, seven nucleotides in length, is formed by complementary 5- and 3-prime terminal sequences with nucleotide contents of only Gs and Cs. The loop domain consists of three antisense sequences; the 5'-CTGTCAGCCATGCCG-3' sequence shown with squares is complementary to target I in Syk mRNA (see Figure 1), the 5'-GCTTCTTGAGGAG-3' sequence shown with triangles is complementary to target II, and the 5'-TGTCTTGCTTTGTC-3' sequence shown with circles is complementary to target III, which is also denoted with circles in Figure 1. For the stem-loop Syk antisense ODN, the three different antisense sequences were tandemly joined in 5' to 3' order for targets I, III, and II to contain minimum intramolecular secondary structure in the loop domain. ●S indicates the phosphorothioate modification; the 5-prime terminus has one phosphorothioate modification, and the 3-prime terminus has two.

the cultured monocytes were incubated with isotype control IgG or anti-FcγRIII IgG (3G8), phagocytosis did not decrease when compared with that of untreated monocytes. However, anti-FcγRII IgG (IV.3) inhibited phagocytosis almost completely (Figure 5). These results further indicate that FcγRII, but not FcγRIII, is expressed on monocytes cultured at 1×10^5 cells/ml and that FcγRIIA mediates phagocytosis in monocytes cultured at this concentration. Monocytes expressing only FcγRII allowed us to study FcγRIIA-mediated phagocytic signaling in monocytes after incubation with the linear and stem-loop Syk antisense ODNs.

Inhibition of Phagocytic Signaling by Stem-Loop Syk Antisense ODN in Monocytes

FcγRIIA mediates phagocytosis in monocytes/macrophages and polymorphonuclear leukocytes (Anderson *et al.*, 1990; Edberg and Kimberly *et al.*, 1992) as well as in epithelial cells and fibroblast transfectants (Mitchell *et al.*, 1994; Indik *et al.*, 1995). Cross-linking of FcγRII leads to tyrosine phosphorylation of several protein substrates, including the receptor itself (Huang *et al.*, 1992; Scholl *et al.*, 1992; Rankin *et al.*, 1993; Ghazizadeh and Fleit, 1994). One of these substrates of potential importance is Syk kinase (Agarwal *et al.*, 1993; Kiener *et al.*, 1993; Greenberg *et al.*, 1994; Pan *et al.*, 1994; Indik *et al.*, 1995a).

Figure 3. Stability of Syk antisense ODNs. One microgram of control phosphodiester ODN, target-I linear Syk antisense ODN, and stem-loop Syk antisense ODN without Lipofectamine (A) or complexed with Lipofectamine (B) were incubated with the following: PBS only (lanes 1, 5, and 9); 10% (lanes 2, 6, and 10) and 33% (lanes 3, 7, and 11) nonheat-inactivated FCS; or 50% human serum (lanes 4, 8, and 12) at 37°C for 16 h. After phenol and chloroform extraction, ODNs were subjected to electrophoresis on a 12% polyacrylamide gel and visualized by ethidium bromide staining. The faint bands (>180 bp) in lanes 4, 8, and 12 are artifacts, as a faint band of the same size was detected with human serum alone.

We investigated the role of Syk kinase in FcγRIIA-mediated phagocytic signaling in monocytes with Syk antisense ODNs. Freshly isolated monocytes were incubated with ODN-liposome complexes; 1.0 μ M each of the linear control or linear Syk antisense ODN or 0.2 μ M each of the stem-loop control or stem-loop Syk antisense ODN was used to form complexes with liposomes. Incubation of monocytes with target-I linear Syk antisense ODN (1.0 μ M) reduced the level of phagocytosis by ~50% (reduction of the phagocytic index [PI] from 220 ± 15.3 to 113 ± 21.4 ; Figure 6A). Target-II and target-III linear Syk antisense ODNs inhibited phagocytosis activity by 43.2% (from 220 ± 15.3 to 125 ± 14.3) and 33.6% (from 220 ± 15.3 to 146

Figure 4. Flow cytometric analysis of monocytes. Monocytes were cultured at cell densities of 1×10^6 (top) or 1×10^5 cells/ml (bottom) for 2 d. Cells were stained with nonspecific control IgG (P3) (a, e), anti-Fc γ RI (32.2) (b, f), anti-Fc γ RII (IV.3) (c, g), or anti-Fc γ RIII (3G8) mAb (d, h) at 4°C for 30 min. Cells were then washed twice with PBS and labeled with FITC-conjugated goat anti-mouse F(ab') $_2$ IgG at 4°C for 30 min.

± 10.3), respectively. Monocytes incubated with the stem-loop Syk antisense ODN ($0.2 \mu\text{M}$) exhibited an even greater reduction in phagocytosis; the PI was reduced from 220 ± 15.3 to 24 ± 7.2 (Figure 6A). We also examined the effect of a mixture of three linear Syk antisense ODNs on phagocytosis in monocytes. Interestingly, when 1×10^5 cells/ml monocytes were treated with a mixture of $0.2 \mu\text{M}$ of each linear Syk antisense ODN, the phagocytic activity was inhibited

Figure 5. Inhibition of phagocytosis by anti-Fc γ RIII IgG. Monocytes cultured for 2 d (1×10^5 cells/ml) were incubated with $50 \mu\text{g/ml}$ isotype control IgG, anti-Fc γ RIII IgG (IV.3), or anti-Fc γ RIII IgG (3G8) at 4°C for 15 min. Phagocytosis of IgG-sensitized red blood cells (EA) was examined by light microscopy and scored as the phagocytic index, PI, number of ingested RBCs/100 cells. Each bar represents the mean $\pm$ SEM of three separate experiments.

by 47.3% (from 220 ± 15.3 to 116 ± 9.3 ; Figure 6A). Neither the scrambled-linear ($1.0 \mu\text{M}$) nor the stem-loop control ($0.2 \mu\text{M}$) ODN affected Fc γ RIIIA-mediated phagocytosis. Similarly, liposomes alone did not reduce Fc γ RIIIA-mediated phagocytosis. Fc γ RIII expression in cultured monocytes did not change with any of the treatments, as measured by flow cytometric analysis. These results indicate that the stem-loop Syk antisense ODN inhibits Fc γ RIIIA-mediated phagocytosis in monocytes more effectively than three linear Syk antisense ODNs or a mixture of them.

We further examined the effect of the stem-loop Syk antisense ODN on the Fc γ receptor-mediated phagocytic signal in monocytes expressing all three classes of Fc γ receptors: Fc γ RI, Fc γ RII, and Fc γ RIII. When monocytes were cultured at 2.5×10^5 cells/ml for 2 d, monocytes expressed all three classes of Fc γ receptors. We and others have previously shown that under similar conditions all three monocyte Fc γ receptors participate in the binding and phagocytosis of IgG-coated cells (Gomez *et al.*, 1989; Anderson *et al.*, 1990; Edberg and Kimberly, 1992; Indik *et al.*, 1994a). When these monocytes (2.5×10^5 cells/ml) were treated with $0.2 \mu\text{M}$ each of the stem-loop control or stem-loop Syk antisense ODN, the phagocytic activity of these monocytes was reduced by $\sim 80\%$ (from a PI of 239 ± 15.8 to 50 ± 6.7 ; Figure 6B).

Syk mRNA and p72^{Syk} Ablated by Stem-Loop Syk Antisense ODN

We examined whether the reduced phagocytosis in monocytes correlated with reduction of Syk-mRNA

Figure 6. Inhibition of phagocytosis by Syk antisense ODNs in monocytes. (A) Monocytes (1×10^5 cells/ml) were incubated twice with complexes containing $2 \mu\text{g}$ of Lipofectamine ($2 \mu\text{g}/\text{ml}$ medium) and $1 \mu\text{g}$ of phosphorothioate ODNs ($0.5 \mu\text{M}$ of the linear control or three linear Syk antisense ODNs or $0.1 \mu\text{M}$ of the mixture of three linear Syk antisense ODNs, the stem-loop control or stem-loop Syk antisense ODN) for 2 d. (B) Monocytes (2.5×10^5 cells/ml) were incubated twice with complexes containing $2 \mu\text{g}$ of Lipofectamine and $1 \mu\text{g}$ of phosphorothioate ODNs ($0.1 \mu\text{M}$ of the stem-loop control or stem-loop Syk antisense ODN) for 2 d. PI, number of ingested RBCs/100 cells. Each bar represents the mean $\pm$ SEM of three separate experiments.

levels. Total RNA was isolated from monocytes (1×10^5 cells/ml) that had been incubated with phosphorothioate ODNs and used to synthesize the first-strand cDNA. Quantitative RT-PCR was performed to determine intracellular levels of Syk mRNA in monocytes. Target-I linear Syk antisense ODN ($1.0 \mu\text{M}$) substantially reduced the level of Syk mRNA. Furthermore, the stem-loop Syk antisense ODN ($0.2 \mu\text{M}$) completely eliminated Syk mRNA (Figure 7A). In contrast, the two scrambled-control ODNs ($1.0 \mu\text{M}$ of the linear control or $0.2 \mu\text{M}$ of the stem-loop control), as well as liposome alone, did not reduce Syk mRNA. The levels of β -actin mRNA were not affected by any of the ODN treatments in monocytes (Figure 7B). The stem-loop Syk antisense ODN also eliminated Syk mRNA in monocytes incubated at 2.5×10^5 cells/ml, whereas treatment with any of the ODNs did not change β -actin mRNA levels in monocytes (Figure 7, C and D). These results indicate that Syk antisense ODNs are able to eliminate specifically Syk mRNA in monocytes. Further, the stem-loop Syk antisense ODN appeared more efficient for targeting Syk mRNA.

We then examined the effect of stem-loop Syk antisense ODN on the intracellular level of tyrosine-phosphorylated $p72^{\text{syk}}$ in monocytes after FcγRII cross-linking and on monocyte Syk expression. Monocytes (1×10^5 cells/ml) incubated with $0.2 \mu\text{M}$ of stem-loop control or Syk antisense ODN were stimulated by FcγRII cross-linking. Cell lysates were immunoprecipitated with $p72^{\text{syk}}$ Ab. Immunoblots were probed with anti-phosphotyrosine mAb 4G10 to detect tyrosine-phosphorylated $p72^{\text{syk}}$ after FcγRII cross-linking. The data demonstrate that, whereas stem-loop control ODN had no effect, the stem-loop Syk

antisense ODN eliminated tyrosine-phosphorylated $p72^{\text{syk}}$ after FcγRII cross-linking (Figure 8A). We also examined whether Syk protein was ablated by the stem-loop Syk antisense ODN. Immunoblotting of whole-cell lysates (2×10^5 cells) was probed with $p72^{\text{syk}}$ Ab to examine intracellular Syk expression. The data demonstrate that stem-loop Syk antisense ODN ablated Syk protein. No effect was shown with stem-loop control ODN (Figure 8B). These results indicate that the stem-loop Syk antisense ODN specifically eliminates $p72^{\text{syk}}$ and that Syk kinase is required for FcγRIIA-mediated phagocytosis in monocytes. Further, because the stem-loop Syk antisense ODN inhibited phagocytosis (Figure 6B) and eliminated Syk mRNA (Figure 7, C and D) in monocytes (2.5×10^5 cells/ml) that express FcγRI and FcγRIII, Syk kinase is also important for phagocytosis mediated by FcγRI and FcγRIIIA.

FcγRII Tyrosine Phosphorylation in Monocytes Treated with Stem-Loop Syk Antisense ODN

We examined the change of FcγRII tyrosine phosphorylation after FcγRII cross-linking in the absence of Syk protein. Monocytes (1×10^5 cells/ml) treated with $0.2 \mu\text{M}$ of stem-loop control or stem-loop Syk antisense ODN were stimulated by FcγRII cross-linking. Cell lysates (5×10^5 cells) were immunoprecipitated with anti-FcγRII mAb (IV.3), and immunoblots were probed with anti-phosphotyrosine mAb 4G10 to detect tyrosine-phosphorylated FcγRII after FcγRII cross-linking (Figure 9A). After this blot was stripped, reprobing with anti-FcγRII mAb II1A5 was performed (Figure 9B). Further, restripping and reprobing with

Figure 7. Effect of Syk antisense ODNs on Syk-mRNA levels in monocytes. (A) Syk mRNA; total RNA was isolated from monocytes (1×10^5 cells/ml) treated twice with complexes containing $2 \mu\text{g}$ of Lipofectamine ($2 \mu\text{g}/\text{ml}$ culture medium) and $1 \mu\text{g}$ of phosphorothioate ODNs ($0.5 \mu\text{M}$ of the linear control or target-I linear Syk antisense ODN or $0.1 \mu\text{M}$ of the stem-loop control or stem-loop Syk antisense ODN) for 2 d. cDNA was synthesized from total RNA with random hexanucleotide primers. PCR was performed with synthesized cDNA as templates with two Syk primers (Syk-H and Syk-M), as described in MATERIALS AND METHODS. PCR products were analyzed by Southern hybridization, and hybridized bands were visualized with chemiluminescent detection reagents. (C) Syk mRNA; total RNA was isolated from monocytes (2.5×10^5 cells/ml) treated twice with complexes containing $2 \mu\text{g}$ of Lipofectamine and $0.1 \mu\text{M}$ of the stem-loop control or stem-loop Syk

Figure 8. Expression of tyrosine-phosphorylated $p72^{\text{syk}}$ and Syk protein in monocytes treated with stem-loop Syk antisense ODN. (A) Unstimulated (–) or $\text{Fc}\gamma\text{RII}$ cross-linked (+) monocytes (5×10^5 cells) treated twice with complexes containing $2 \mu\text{g}$ of Lipofectamine ($2 \mu\text{g}/\text{ml}$ culture medium) and $0.1 \mu\text{M}$ of the stem-loop control or stem-loop Syk antisense ODN for 2 d were lysed and immunoprecipitated with $p72^{\text{syk}}$ Ab. Proteins were resolved on 9% SDS-PAGE. Immunoblots were probed with anti-phosphotyrosine mAb 4G10. (B) Monocytes (1×10^5 cells/ml) treated with $0.2 \mu\text{M}$ of stem-loop control or stem-loop Syk antisense ODN were washed in ice-cold PBS and lysed. Cell lysates (2×10^5 cells) were separated by 9% SDS-PAGE, and immunoblots were probed with $p72^{\text{syk}}$ Ab. Blots were visualized with enhanced chemiluminescence. The arrow indicates the position of the 72-kDa Syk protein. Molecular weight markers are shown at the left.

$p72^{\text{syk}}$ Ab were performed to examine Syk protein expression (Figure 9C). The data demonstrate that, after $\text{Fc}\gamma\text{RII}$ cross-linking, $\text{Fc}\gamma\text{RII}$ tyrosine phosphorylation was not changed in the absence of Syk protein (Figure 9). These data indicate that Syk kinase is required for the phagocytic signal mediated by $\text{Fc}\gamma\text{RIIA}$ and that $\text{Fc}\gamma\text{RII}$ cross-linking leads to tyrosine phosphorylation of $\text{Fc}\gamma\text{RII}$ independent of Syk kinase.

(Figure 7 cont.) antisense ODN for 2 d. cDNA was synthesized from total RNA with random hexanucleotide primers. PCR was performed with synthesized cDNA as templates with two Syk primers (Syk-H and Syk-M). (B and D) β -Actin mRNA; PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

DISCUSSION

In these studies we demonstrate that a stem-loop antisense ODN is effective in eliminating the expression of Syk mRNA and protein and that Syk kinase is required for the phagocytic signal mediated by Fc γ receptors. We further show that Fc γ RII tyrosine phosphorylation is independent of Syk kinase in Fc γ RIIA-mediated signaling. Although a role for Syk kinase in Fc γ receptor signaling has been suggested by previous studies from our and other laboratories, a requirement for Syk kinase and its role in Fc γ receptor tyrosine phosphorylation previously has not been established. This is of particular significance, because we have previously observed that the tyrosine kinase Src is not required for phagocytic signaling by Fc γ RIIA (Hunter *et al.*, 1993).

Cultured monocytes express all three Fc γ receptor classes (Ravetch and Kinet, 1991; van de Winkel and Anderson, 1991; McKenzie and Schreiber, 1994; Indik *et al.*, 1995b). However, monocytes cultured at a density of 1×10^5 cells/ml fail to express Fc γ RI and Fc γ RIII and express only Fc γ RII. Although the precise mechanism for this observation is uncertain, at a low cell density less cell/cell interaction and/or an insufficient quantity of monocyte-secreted cytokines may reduce the expression of Fc γ RI and Fc γ RIII. Increased expression of Fc γ RI and Fc γ RIII at conditions of high monocyte/macrophage density may result in enhanced phagocytosis and/or increased release of intracellular mediators.

Although neither of the Syk antisense ODNs affected Fc γ RII expression, they inhibited phagocytosis. Shown by RT-PCR and immunoblotting, the effect of Syk antisense ODN on phagocytosis was due to degradation of specific mRNA and the resulting protein ablation as Syk mRNA and p72^{Syk} were eliminated. That there was no evidence for nonspecific effects of antisense ODN may be due to the low concentrations of antisense ODN used in our experiments. It should be noted that the concentrations of antisense ODNs that we used were 10- to 100-fold lower than those described for many other antisense ODN applications. After treatment with the stem-loop Syk antisense ODN, a small degree of residual phagocytic activity remained even when Syk mRNA or p72^{Syk} was undetected. This phagocytic activity may be due to a minor, Syk-independent phagocytic signal.

We also examined the effect of the stem-loop Syk antisense ODN on HEL cells, a megakaryocyte/platelet-like cell line (King *et al.*, 1992) that expresses Fc γ RII as its only Fc γ receptor (Rappaport *et al.*, 1991). The stem-loop Syk antisense ODN also reduced phagocytosis and the level of Syk mRNA in HEL cells. These results indicate that the effect of stem-loop Syk antisense ODN is not cell-type specific and that Syk kinase

Figure 9. Fc γ RII tyrosine phosphorylation in monocytes treated with stem-loop Syk antisense ODN. (A) Unstimulated (–) or Fc γ RII cross-linked (+) monocytes (5×10^5 cells) treated twice with complexes containing 2 μ g of Lipofectamine (2 μ g/ml culture medium) and 0.1 μ M of the stem-loop control or stem-loop Syk antisense ODN for 2 d were lysed and immunoprecipitated with anti-Fc γ RII mAb (IV.3). Proteins were resolved on 11% SDS-PAGE. Immunoblots were probed with anti-phosphotyrosine mAb 4G10. The blot from A was stripped and reprobed with anti-Fc γ RII mAb II1A5 (B) or with p72^{Syk} Ab (C). Blots were visualized with enhanced chemiluminescence. The arrow and asterisk indicate the position of the 72-kDa Syk protein and the 40-kDa Fc γ RII protein, respectively. Molecular weight markers are shown at the left.

is also required for Fc γ RIIA-mediated phagocytosis in a megakaryocyte/platelet-like cell line.

The stem-loop Syk ODN inhibited phagocytosis in monocytes cultured at 2.5×10^5 cells/ml expressing all three classes of Fc γ receptors (Figure 6B), suggesting that the phagocytic signal induced by Fc γ RI and Fc γ RIIA also requires Syk kinase. These data extend our observations that the transfection of Syk kinase enhances Fc γ RI and Fc γ RIIA-mediated phagocytosis (Indik *et al.*, 1995a).

Although three isoforms of Fc γ receptors can mediate the phagocytosis of IgG-coated cells, their requirements for phagocytosis differ. Fc γ RI and Fc γ RIIA require a γ subunit and its cytoplasmic immunoreceptor tyrosine-activation motif (ITAM) for phagocytosis

(Park *et al.*, 1993a,b; Indik *et al.*, 1995a). On the other hand, Fc γ RIIA mediates phagocytosis through a unique conserved ITAM sequence present in its own cytoplasmic domain and, thus, does not require the γ subunit (Indik *et al.*, 1994b; Mitchell *et al.*, 1994). Transfection of Syk kinase enhances phagocytosis mediated by Fc γ RI and Fc γ RIIIA seven- to tenfold but enhances phagocytosis by Fc γ RIIA two- to threefold in COS-1 cells (Indik *et al.*, 1995b). In the absence of Syk transfection, Fc γ RIIA-mediated phagocytosis is much more efficient than phagocytosis by the γ subunit of Fc γ RI and Fc γ RIIIA (Indik *et al.*, 1995b). The low level of endogenous Syk kinase in COS-1 cells is likely sufficient for Fc γ RIIA signaling. However, it is uncertain as to precisely how Syk kinase is involved in Fc γ RIIA-mediated signaling. In this study we demonstrate that Fc γ RII tyrosine phosphorylation is independent of Syk kinase (Figure 9). The data indicate that protein tyrosine kinases other than Syk are involved in tyrosine phosphorylation of Fc γ RII. Of interest is that the pattern of protein tyrosine phosphorylation after Fc γ RII cross-linking does not seem to change significantly in the absence of Syk protein. The use of Syk antisense ODNs should allow us in future studies to define the substrate(s) of Syk kinase. It is possible that Fc γ RIIA-mediated phagocytosis may involve downstream proteins that are not tyrosine phosphorylated. Alternatively, anti-phosphotyrosine immunoblots may not be sufficiently sensitive to discriminate decreased protein tyrosine phosphorylation in the absence of Syk.

Antisense ODNs offer the potential to reduce or ablate a specific mRNA. Among the factors that affect the efficacy of antisense ODNs are the stability of the antisense ODNs, their delivery into the cell cytoplasm, and their accessibility to the target mRNA. We have modified Syk antisense ODN in three steps to address these issues. First, one phosphodiester link at the 5-prime and two at the 3-prime termini were modified with phosphorothioates. Second, we have designed a stem-loop structure to protect the antisense sequence in the loop domain from nucleases. The stem has complementary terminal sequences with only Gs and Cs. The loop domain has three antisense sequences targeting different sites of Syk mRNA. mRNA forms secondary structures by intramolecular hybridization, and mRNA secondary structures may inhibit access of antisense ODNs to target sequences. To identify sequences with open structures that may provide better access for antisense ODNs, we scanned the entire Syk-mRNA sequence with an RNA secondary-structure prediction program. Syk mRNA was scanned in three staggered frames, and the most open sequences with minimum secondary structures were carefully chosen. Our study did not seek to investigate the advantage in targeting open mRNA sequences, but the fact that all three linear Syk antisense ODNs inhibited phagocytosis

suggests that open substrate sequences may be readily accessible to antisense ODNs. Because stem-loop antisense ODN reduced the phagocytic signal more dramatically than the mixture of three linear antisense ODNs (Figure 6A), the higher efficacy of the stem-loop Syk antisense ODN may be due to better stability from nuclease digestion. Third, Syk antisense ODNs were also complexed with cationic liposomes to improve delivery to the cells. The stability of the stem-loop Syk antisense ODN markedly improved when complexed with liposomes, providing an additional benefit for the use of ODN-liposome complexes. Our stem-loop antisense ODN approach has been used successfully to abrogate the expression of other gene products. We have used a stem-loop antisense ODN directed at the Fc γ RIIIA γ subunit mRNA. With the use of peripheral blood monocytes and the stem-loop γ -chain antisense ODN, the monocyte γ -chain message, assessed by RT-PCR, was decreased by >80%. Liposomes can be delivered to the reticuloendothelial system, for which monocytes/macrophages are a major residential cell population. The complex of liposome-stem-loop Syk antisense ODN may be advantageous for use as a therapeutic agent(s) for immunologic disorders requiring down-regulation of Fc γ receptor-mediated function in monocytes/macrophages. Syk kinase is also associated with Fc ϵ RI and with the B-cell antigen receptor (Hutchcroft *et al.*, 1992a,b). The stem-loop Syk antisense ODN may also prove useful for investigating intracellular signaling events through these receptors and for developing a therapeutic agent(s) to modulate the signals mediated by these receptors.

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