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1α, 25-Dihydroxy-Vitamin D3 Alters Syk Activation Through FcγRII in Monocytic THP-1 Cells

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Abstract In monocytes and macrophages, activation of the tyrosine kinase Syk is an essential step in the biochemical cascade linking aggregation of receptors for immunoglobulin G (FcγR) to initiation of effector functions. An increase in Syk activation during differentiation of myeloid cells by different agents has been reported. We studied the activation state of Syk in response to FcγRII crosslinking in monocytic cells before and after in vitro differentiation with 1α, 25-dihydroxy-vitamin D3. We show here that while in undifferentiated THP-1 cells clustering of FcγRII induces significant phosphorylation and activation of Syk, in THP-1 cells differentiated in vitro by 1α, 25-dihydroxy-vitamin D3, FcγRII crosslinking induced a decrease in Syk activity. In vitro differentiation did not induce changes in the expression of FcγRII isoforms. The observed effect on Syk activation though FcγRII could be mediated by differentiation-induced changes in the expression and basal activation level of Syk, as well as changes in the association of Syk with the tyrosine phosphatase SHP-1. These results suggest that the biochemical signaling pathways induced by FcγRII could be dependent on the differentiation state of the cell. J. Cell. Biochem. 89: 1056–1076, 2003.

Key words: mononuclear phagocytes; IgG receptors; tyrosine kinase; monocyte differentiation

Macrophages and their blood precursors, monocytes, play important roles in host defense and homeostasis. Monocytes and macrophages express receptors for the Fc portion of immunoglobulin G (FcγR), which are members of the immunoglobulin superfamily [Hunkapillar and Hood, 1989]. Three classes of biochemically distinct FcγRs have been described: FcγRI, FcγRII, and FcγRIII [Ravetch and Kinet, 1991; Unkeless et al., 1992], and each class includes several isoforms. FcγRI is a high-affinity receptor that binds monomeric IgG, while FcγRII and FcγRIII are low-affinity receptors that bind multimeric immune complexes [Ravetch and Kinet, 1991; Cassel et al., 1993; Daeron, 1997].

In humans, both FcγRI and FcγRII are each encoded by three genes (A, B, and C) located at chromosome 1 (q21–23), whereas two genes (A and B) code for FcγRIII [Ravetch and Kinet, 1991; Cassel et al., 1993; Daeron, 1997]. FcγRI expressed in the membrane of hematopoietic cells is encoded in the gene A and is a transmembrane receptor with three extracellular Ig-like domains. Genes B and C encode secreted forms of the receptors with only two Ig-like domains [Ernst et al., 1992].

FcγRII A, B, and C genes encode transmembrane receptors bearing two highly homologous extracellular domains, but the receptors encoded by FcγRIIB differ considerably in their cytoplasmic domains from those encoded by FcγRIIA and FcγRIIC. FcγRIIA originates two transcripts: $Fc\gamma RIIa_1$ (which encodes a transmembrane receptor) and $Fc\gamma RIIa_2$ (which lacks the transmembrane exon and thus generate soluble IgG-binding factor [Rappaport et al., 1993]. FcγRIIB generates three transcripts ($Fc\gamma RIIb_1$, $Fc\gamma RIIb_2$, and $Fc\gamma RIIb_3$) [Cassel

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et al., 1993]. FcγRIIC produces four different transcripts (FcγRIIc$_{1\text{-}4}$) in NK cells; of these, FcγRIIc$_1$ is the form expressed in monocytes and macrophages [Mates et al., 1998]. The cytoplasmic tails of FcγRIIA$_1$ and FcγRIIC$_1$ are identical and contain a sequence motif, termed the immunoreceptor–tyrosine-based activation motif (ITAM; consensus sequence: D/EX$_2$YX$_2$ LX$_{7-12}$YX$_2$L/I), found in several signaling chains of antigen receptors and immunoglobulin receptors [Reth, 1989], including the γ-chains associated with FcγRI and FcγRIIIA [Park et al., 1993; Indik et al., 1994]. FcγRIIB$_1$ and FcγRIIB$_2$ are identical except for a 19-amino-acid insert in the cytoplasmic tail of FcγR IIB$_1$. Both receptors have a 13-mer containing the consensus I/VxYxxL/V sequence found in many inhibitory receptors known as immunoreceptor tyrosine-based inhibition motif (ITIM) [Daeron et al., 1995; Cambier, 1997]. Coaggregation of the ITIM-containing FcγRIIB molecules with ITAM containing immune receptors such as the BCR, induces phosphorylation of the tyrosines within the ITIM motif. Once phosphorylated, the ITIM motifs can recruit SH2 domain-containing protein phosphatases such as SHP-1 and SHP-2 as well as the phosphatidylinositol 5′-phosphatases SHIP-1 and SHIP-2 [D'Ambrosio et al., 1995, 1996; Ono et al., 1996; Sato and Ochi, 1998]. The recruitment and activation of these phosphatases have been shown to negatively regulate signaling by ITAM-containing receptors.

Crosslinking of FcγRs by immune complexes or IgG-opsonized particles induces phosphorylation of tyrosine residues within the cytoplasmic domains of the receptors or associated subunits [Santana et al., 1996]. The first intracellular enzymes known to be activated after FcγR crosslinking are tyrosine kinases of the Src family [Jouvin et al., 1994]. Src kinase activation results in a rapid and transient phosphorylation of the ITAMs on either the cytoplasmic domain of FcγRIIA or the γ-chains associated with FcγRI and FcγRIIIA [Park et al., 1993; Indik et al., 1994, 1995]. FcγRs crosslinking also results in an increase in tyrosine phosphorylation and activation of Syk [Agarwal et al., 1993; Kiener et al., 1993]. Syk is a protein tyrosine kinase of the Syk/ZAP-70 family, composed of two N-terminal Src homology domains and a C-terminal catalytic domain [Bolen and Brugge, 1997; Kiefer et al., 1998]. In monocytes and macrophages, FcγR aggregation induces Syk association with the phosphorylated ITAM motifs of the γ-chain of the FcγRI and FcγRIIIA and of the cytoplasmic domain of FcγRIIA. Once associated, Syk becomes phosphorylated on tyrosine, is activated, and catalyzes the phosphorylation of multiple substrates, including other FcγRs ITAMs and downstream effectors [Greenberg et al., 1994; Rowley et al., 1995]. Syk activation has been shown to be a crucial step in FcγR mediated signaling, since blocking Syk activation, either by the specific inhibitor piceatannol, or by antisense oligonucleotides, completely abolishes downstream signaling stimulated through FcγRs [Matsuda et al., 1996; Pain et al., 2000].

Syk is not only important in signaling through a variety of receptors in both lymphoid and myeloid cells [Chan et al., 1994; Turner et al., 2000], but it has also been shown to play an essential role in the development of B cells [Cheng et al., 1995; Turner et al., 1995] and a subset of T cells [Turner et al., 1995; Mallick-Wood et al., 1996]. The role of Syk during differentiation of myeloid cells has not been studied in detail. An increase in tyrosine phosphorylation and catalytic activity of Syk has been observed during in vitro differentiation of HL-60 cells into granulocytes induced by all-*trans* retinoic acid [Qin and Yamamura, 1997].

Monocyte to macrophage differentiation is a complex process that can follow distinct pathways depending on the signals acting on the cell, thus producing the high degree of functional heterogeneity of mature macrophages. This functional heterogeneity includes almost all macrophage functions: ability as APC, secretion, and effector functions including the responses mediated by FcγRs [Adams and Hamilton, 1992]. Given the essential role of Syk in signaling through FcγRs and the possible involvement of Syk in the differentiation of cells expressing these receptors, we were interested in determining if the differentiation along a particular monocyte-macrophage pathway could affect the activation of Syk in response to FcγR crosslinking. As a model system, we studied the activation of Syk in response to FcγR crosslinking in the human monocytic cell line THP-1, before and after it was induced to differentiate with 1α,25-dihydroxyvitamin D$_3$ (VD3). This metabolite of Vitamin D has multiple effects on the differentiation and function of hematopoietic cells in vivo [Bouillon et al., 1995], and has been shown to promote the in vitro differentiation of monocytic cell lines into a more macrophage-like

phenotype [Choudhuri et al., 1990; Kreutz and Andreesen, 1990; Schwende et al., 1996].

We found that in undifferentiated THP-1 cells, FcγRII crosslinking induces significant Syk activation. However, in THP-1 cells differentiated by VD3, the phosphorylation level and activation state of Syk following FcγRII crosslinking was greatly reduced. The changes that we found in the expression of FcγRII isoforms do not seem to be related to this effect. However, we found that differentiation with VD3 induces changes in the association of Syk with the protein phosphatase SHP-1, and this can explain the inhibition of Syk activation after FcγRII crosslinking.

These results demonstrate that the biochemical signaling pathways induced by crosslinking of FcγRII are dependent on the differentiation status of the cell.

MATERIALS AND METHODS

Reagents and Antibodies

Fetal bovine serum (FBS) and Protein A-Sepharose beads were purchased from Gibco Laboratories (Grand Island, NY). The 1-α,25-dihydroxy-vitamin D_3 (1α, 25-(OH) $_2$ $VitD_3$), VD3 was from CALBIOCHEM (La Jolla, CA). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO). 2,4,6-Trinitrobenzene sulphonic acid (TNBS) for sensibilization of sheep erythrocytes was from Eastman Kodak Co. Murine monoclonal anti-human FcγRI (32.2) and FcγRII (IV.3) mAbs were purified in our laboratory from supernatants of the corresponding hybridomas obtained from ATCC. Fab fragments were prepared from the purified antibody with Immobilized Pepsin (Pierce), following the manufacturer's instructions. Murine monoclonal anti-human FcγRIIIA (3G8) was from Zymed (San Francisco, CA). Anti-Syk (SC-573), anti-SHP-1 (SC-287), and anti-phosphotyrosine antibodies (PY-20, SC-508, and PY-20-HRP, SC-508*HRP*) were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG $F(ab)'_2$ was from Zymed (62–6120); goat anti-mouse IgG-HRP was from Jackson Immuno Research (Amish, PA). Anti-CD11b monoclonal antibody (2LPM19c) (M 0747) was from DAKO Corporation (Carpinteria, CA). Mouse monoclonal DNP-specific antibodies 2C5 (IgG_1) and 4F8 (IgG_{2b}) used as isotype controls for cytofluorometry and as opsonizing antibodies in the phagocytosis assay were produced in our laboratory from culture supernatants of the corresponding hybridomas. Goat anti-mouse IgG-FITC was from Zymed. Myelin basic protein (MBP) was kindly donated by Dr. Janet Oliver (University of New Mexico, Albuquerque, NM). γ-^{32}P-ATP was from New England Nuclear (Beverly, MA).

Reagents for RNA isolation and RT-PCR were purchased from Gibco-BRL, Inc. (Gaithersburg, MD) and Sigma Chemical Corp. (St. Louis, MO). Taq DNA polymerase was purchased from Perkin-Elmer (Branchburg, NJ).

Flow Cytometry

THP-1 cell suspensions (0.5×10^6 cells/ml) in PBS with 5% FBS and 0.01% sodium azide were incubated with 10 μg/ml of one of the following primary murine monoclonal antibodies: anti-FcγRI (32.2), anti-FcγRII (IV.3), anti-FcγRIIIA (3G8), anti-CD11b (2LPM19c), anti-DNP IgG_1 (2C5), or anti-DNP IgG_{2b} (4F8) for 60 min at 4°C. After washing, cells were incubated in the dark for 90 min with 0.45 μg/ml FITC-labeled goat anti-mouse IgG at 4°C. After washing, the cells were fixed for 30 min in 0.3% paraformaldehyde, followed by three washes with PBS. The stained cells were analyzed in a FACscan cytometer (Becton Dickinson, San Jose, CA).

Cell Culture and In Vitro Differentiation

The monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium (GIBCO-BRL) supplemented with 10% (v/v) heat-inactivated FBS, 1 mM MEM Sodium pyruvate solution, 2 mM MEM non-essential amino acids solution, 0.1 mM of L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Differentiation was induced by culturing THP-1 cells (3×10^6 cells/ml) in the presence of 100 nM of 1α,25-dihydroxy-vitamin D_3 for 72 h. Cell differentiation was confirmed by changes in cell morphology, as well as increases in membrane expression of complement receptor type 3 (CR3). Microscopic phase-contrast images of undifferentiated and VD3-differentiated cells were obtained in an Axiovert 25 (Carl Zeiss) inverted microscope attached to a photographic camera.

Cell Stimulation and Immunoprecipitation

THP-1 cell suspensions (1×10^7 cells/ml) were maintained in serum-free RPMI-1640 medium

for 10 min on ice, previous to the incubation with 10 μg/ml of Fab fragments of mouse anti-FcγRI (32.2) or anti-FcγRII (IV.3) mAb, for 10 min on ice. The cell suspension was then centrifuged at 15,000*g* for 1 min at 4°C, and the supernatant was discarded. To induce FcγR aggregation, the cells were resuspended in 1.0 ml of fresh medium (without FBS) containing 10 μg/ml of $F(ab)'_2$ fragments of rabbit anti-mouse IgG, for the indicated times at 37°C. Stimulation was stopped by addition of 500 μl of ice-cold TBS (10 mM Tris-HCl, 100 mM NaCl, pH 7.4) and the cells were pelleted by centrifugation at 4°C. The supernatant was discarded and the cells were lysed in 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenyl-methyl sulfonyl fluoride, 10 mM NaF and 1 μg/ml of Pepstatin A, Leupeptin and Aprotinin) and kept on ice for 15 min. Lysates were clarified by centrifugation at 15,000*g* for 15 min at 4°C. Protein concentration in cell lysates was determined by the DC Protein Assay (Bio-Rad, Hercules, CA), following the manufacturer's instructions. For immunoprecipitation, the clarified lysates were incubated for 3 h at 4°C with anti-Syk antibodies prebound to protein A-Sepharose beads. Immunoprecipitates were washed three times with washing buffer (of identical composition as lysis buffer but with 0.1% Triton X-100), and used for in vitro kinase assays, or they were boiled in Laemmli sample buffer and separated on 10% SDS–PAGE for immunoblotting.

Where indicated, cells were incubated before stimulation with 100 μM pervanadate for 5 min at 37°C. Pervanadate was generated by mixing 1 ml of 20 mM Na_3VO_4 with 330 μl of 30% H_2O_2 and incubating for 5 min at room temperature, obtaining a solution of 6 mM pervanadate.

Immunoblotting

Immunoprecipitates or whole cell lysates were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 1% BSA and 3% non-fat dry milk in low-salt Tween-20-Tris-buffered saline (T-TBS) (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.1% Tween-20) overnight at 4°C. This was followed by incubation with primary antibody (anti-phosphotyrosine (PY-20), anti-Syk or anti-SHP-1) for 90 min at room temperature. After washing, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase in T-TBS for 1 h at room temperature. For chemiluminiscent detection, blots were treated with Super Signal ECL kit (Pierce) according to the manufacturer's instructions, and exposed to X-ray films. Digitalized images of the developed films used to capture the chemiluminiscent signals were obtained with the Gel-Doc 2000 System (Bio-Rad) and analyzed with the Bio-Rad Quantity One software. Where indicated, membranes were stripped and reblotted with a different primary antibody. For stripping, membranes were incubated in 0.1 M glycine (pH 2.5) for 1 h at 60°C. After washing, the membranes were blocked and incubated with primary and secondary antibodies and processed as described above.

In Vitro Kinase Assays

The washed immunoprecipitates obtained as described above, were incubated in 50 μl of kinase buffer (50 mM HEPES-sodium hydroxide pH 8.0, 10 mM Na_3VO_4, 50 mM manganesium acetate, 150 mM NaCl, 10 μCi [γ-^{32}P]-ATP, and 2.5 μg/ml MBP) for 10 min at 37°C. After washing three times with 1 ml of kinase buffer (without [γ-^{32}P]-ATP), the immunoprecipitates were boiled in SDS–PAGE sample buffer and resolved on 12.5% SDS–PAGE gels. The dried gels were exposed at −70°C on X-ray films.

Phagocytosis Assay

Sheep RBC were kept at 4°C in Alsever's solution for a maximum of 4 weeks until used. They were washed in $DGVB^+$ and derivatized with 2,4,6-trinitrobenzene sulphonic acid, sodium salt (TNBS) by incubating 1.0 ml of packed SRBC with 12.44 mg of TNBS in 7 ml of borate buffer with gentle shaking and protected from light for 10 min at room temperature. The sensitized RBC were washed two times with $DGVB^+$ and once with RPMI medium without FBS. Fifty microliters of a 2% suspension of sensitized RBC in RPMI were added to each well of a 96-well V-bottom plate containing 100 μl of serial 1:2 dilutions of anti-DNP 4F8 antibody. The plates were incubated at room temperature for 90 min. The hemagglutination titer was determined as the lowest concentration of anti-DNP monoclonal 4F8 antibody that produced visible RBC agglutination. Opsonization of RBC with anti-DNP IgG was carried out by incubating a 1% suspension of sensitized RBC in RPMI with a sub-hemagglutination dilution of anti-DNP IgG at room temperature for 60 min. The

unbound antibodies were removed by centrifugation. For the phagocytosis assay, 320 μl of a suspension of THP-1 cells (1×10^6/ml) was incubated with 60 μl of anti-DNP opsonized or non-opsonized RBC for 2 h at 37°C in a 5% CO_2 humidified incubator. The cells were then washed three times with PBS to remove unbound RBCs. Non-internalized RBCs were lysed with 0.2% PBS for 30 s. Phagocytosis assays were performed in triplicate. RBC ingestion by THP-1 cells was examined by light microscopy by an observer who was blind to the treatment conditions. The results are expressed as the phagocytic index (number of ingested RBC by 100 cells).

RNA Isolation, Reverse Transcription (RT) and PCR

Total RNA was isolated from THP-1 cells by the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction using TRIzol (Gibco-BRL, Inc.) [Chomczynski and Sacchi, 1987]. RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1.1% denaturing agarose gels in the presence of 2.2 M formaldehyde. Total RNA was reverse transcribed to synthesize single strand cDNA as previously described [Camacho-Arroyo et al., 1996]. Ten microliters of RT reaction were subjected to PCR in order to simultaneously amplify FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, and β-actin genes, the latter used as an internal control. The sequences of the specific primers used for amplification are given in Table I. The 50 μl PCR reaction included: 10 μl of previously synthesized cDNA and 40 μl of a mixture containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 2.5 units of Taq DNA polymerase. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. After the initial denaturation step at 95°C for 5 min, 30 cycles were carried out for PCR amplification. The cycle profile for all genes amplification was: 95°C, 1 min; 60°C, 1 min; and 72°C, 1 min. A final extension cycle was performed at 72°C for 5 min. The number of cycles performed was within the exponential phase of the amplification process. Twenty-five microliters of PCR products were separated on 2% agarose gels and stained with ethidium bromide. The image was captured under a UV transilluminator on Type 665 negative film (Polaroid Co., Cambridge, MA). In each experiment, the amplification and analysis of the products of each gene were carried out in parallel. The images were captured in a Scan Jet 3C scanner (Hewlett-Packard) and the intensities of the individual bands were quantified using a Scand Primax 600p (Colorado), and Scion Image software. To get a semi-quantitative estimation of the mRNAs for each FcγR isoform, the intensity of the band corresponding to the amplification product was normalized to the intensity of the β-actin band. Thus, the relative expression level of each FcγR isoform is expressed as the ratio of intensities of its corresponding band to β-actin band.

TABLE I. Sequences of FcγRI and FcγRII-Specific Primers for RT-PCR

Receptor	S, sense; AS, antisense	Sequence
FcγR I	S	5′TGAATACAGGGTGCCAGAGAG 3′
	AS	3′AGAAGTAAAGCTTGCAAACCA 5′
FcγR IIA	S	5′CACGCTGTTCTCATCCAAG 3′
	AS	3′ATTCCCCTCTTTTTGTCATCC 5′
FcγR IIB_1	S	5′ACAACAATGACAGCGGGGA 3′
FcγR IIB_2	AS	3′GGTGCATGAGAAGTGAATAG 5′
FcγR IIC	S	5′TCCATCCCACAAGCAAACCA 3′
	AS	3′TTTATCATCGTCAGTAGGTGC 5′

Statistical Analysis

Data were analyzed by using a one way analysis of variance (ANOVA) followed by a Student's *t*-test. Prism 2.01 program (Graph Pad, CA) was used for calculating probability values.

RESULTS

VD3-Induced Differentiation of THP-1 Cells does not Alter Membrane Expression of FcγRs

In vitro treatment of THP-1 cells with the active metabolite of Vitamin D3 has been shown to induce some differentiation related changes [Kreutz and Andreesen, 1990; Schwende et al., 1996]. To determine whether VD3 treatment modulates the surface expression of FcγRs in THP-1 monocytic cells, cells were incubated in RPMI-1640—10% FBS for 72 h with or without 100 nM of VD3. Surface expression of CD11b/CD18 (CR3), FcγRI, FcγRII, and FcγRIII was evaluated by cytofluorometry using mAbs 2LP-M19c, 32.2, IV.3, and 3G8 specific for CD11b, FcγRI, FcγRII, and FcγRIII, respectively. VD3

treatment induced differentiation-associated changes, such as changes in morphology and adherence of the cells to the culture flasks, as well as an increase in CD11b/CD18 surface expression. In contrast, treatment with VD3 for 72 h did not significantly alter membrane expression of FcγRI, FcγRII, or FcγRIII (Fig. 1).

FcγRII Crosslinking Induces Syk Phosphorylation

Activation of the tyrosine kinase Syk is an essential step in the biochemical cascade initiated by FcγR crosslinking [Kiener et al., 1993; Durden and Liu, 1994]. To measure the level of Syk phosphorylation induced after crosslinking FcγRII in THP-1 cells, the cells were first incubated at 4°C with saturating amounts of Fab fragments of anti-FcγRII mAb for 10 min, followed by crosslinking of the cell bound fragments with increasing concentrations of $F(ab)'_2$ fragments of rabbit anti-mouse IgG at 37°C. After the stimulation, cells were lysed, Syk was immunoprecipitated from equivalent amounts of total protein lysates with anti-Syk antibodies, and the level of tyrosine phosphorylation of Syk was assessed by anti-phosphotyrosine immunoblotting (Fig. 2A). To account for possible differences in the amount of Syk immunoprecipitated, the same blot was stripped and reprobed with anti-Syk antibody. The results show that FcγRII crosslinking induces a significant increase in the level of Syk phosphorylation. In the experimental conditions used, Syk phosphorylation increases as the amount of secondary antibody is increased, reaching a maximum at 10 μg/ml of secondary antibodies (Fig. 2A). The maximal increase in tyrosine phosphorylated proteins after FcγRII crosslinking is seen at 3 min of stimulation as shown in Figure 2B. Using these experimental conditions, we performed experiments in which THP-1 cells were stimulated by crosslinking FcγRII and the level of phosphorylation of Syk was determined by immunoprecipitating Syk, resolving the immunoprecipitates in SDS–PAGE, transferring the resolved proteins to nitrocellulose membranes, and sequentially blotting the membranes with anti-PY and anti-Syk antibodies. Figure 2C shows a representative experiment and Figure 2D shows the average of three independent experiments in which the level of Syk phosphorylation for each condition was determined as the ratio of anti-PY signal to anti-Syk signal of the relevant bands.

Effect of VD3-Induced Differentiation on Syk Phosphorylation Levels After FcγR Crosslinking

It has been reported that the activity of Syk can be modulated during the differentiation of HL-60 promyelocytic leukemia cells [Qin and Yamamura, 1997]. To determine whether differentiation of THP-1 cells induced by VD3 treatment has any effect on the amount of Syk in the cells, we performed anti-Syk inmunoblots in lysates of cells treated with VD3 for 0, 24, 48, and 72 h. The blot was stripped and re-probed with anti-actin antibody and the results are expressed as the ratio of the anti-Syk to the anti-actin band for each sample. The results showed a time-dependent increase in Syk levels in THP-1 cells treated with 100 nM VD3 (Fig. 3).

To determine whether differentiation affects Syk activation after FcγRII crosslinking, we compared the level of Syk phosphorylation induced through FcγRII in THP-1 cells treated or not with VD3 (100 nM) for 72 h. Both non-treated and VD3-treated THP-1 cells were stimulated through FcγRII as described above. After stimulation, cells were lysed and the levels of Syk phosphorylation were compared in anti-Syk immunoprecipitates. In untreated cells, FcγRII crosslinking induced a significant increase in phosphorylation of Syk (Fig. 4A, lanes 1 and 2). VD3 treatment by itself induced an increase in the basal level of Syk phosphorylation (Fig. 4A, lane 3). Surprisingly, in VD3 treated cells the level of Syk phosphorylation after FcγRII crosslinking was significantly lower than that observed in unstimulated VD3-treated cells (Fig. 4A, compare lanes 3 and 4). The lower panel of Figure 4A shows the results of three independent experiments performed on different batches of THP-1 cells. The level of Syk activation induced by FcγRII crosslinking in VD3-treated and untreated cells was also assessed by determining the in vitro kinase activity of immunoprecipitated Syk on an exogenous substrate (MBP). In untreated cells, FcγRII crosslinking induced an increase in Syk activity (Fig. 4B, lane 2). Similar to what was observed in the level of Syk phosphorylation, VD3 treatment increased the basal level of Syk activity, and this basal level of activity decreased after FcγRII crosslinking (Fig. 4B compare lanes 3 and 4). Thus, while in undifferentiated THP-1 cells FcγRII crosslinking induces Syk phosphorylation and activation, after differentiation induced by VD3 the response to FcγRII

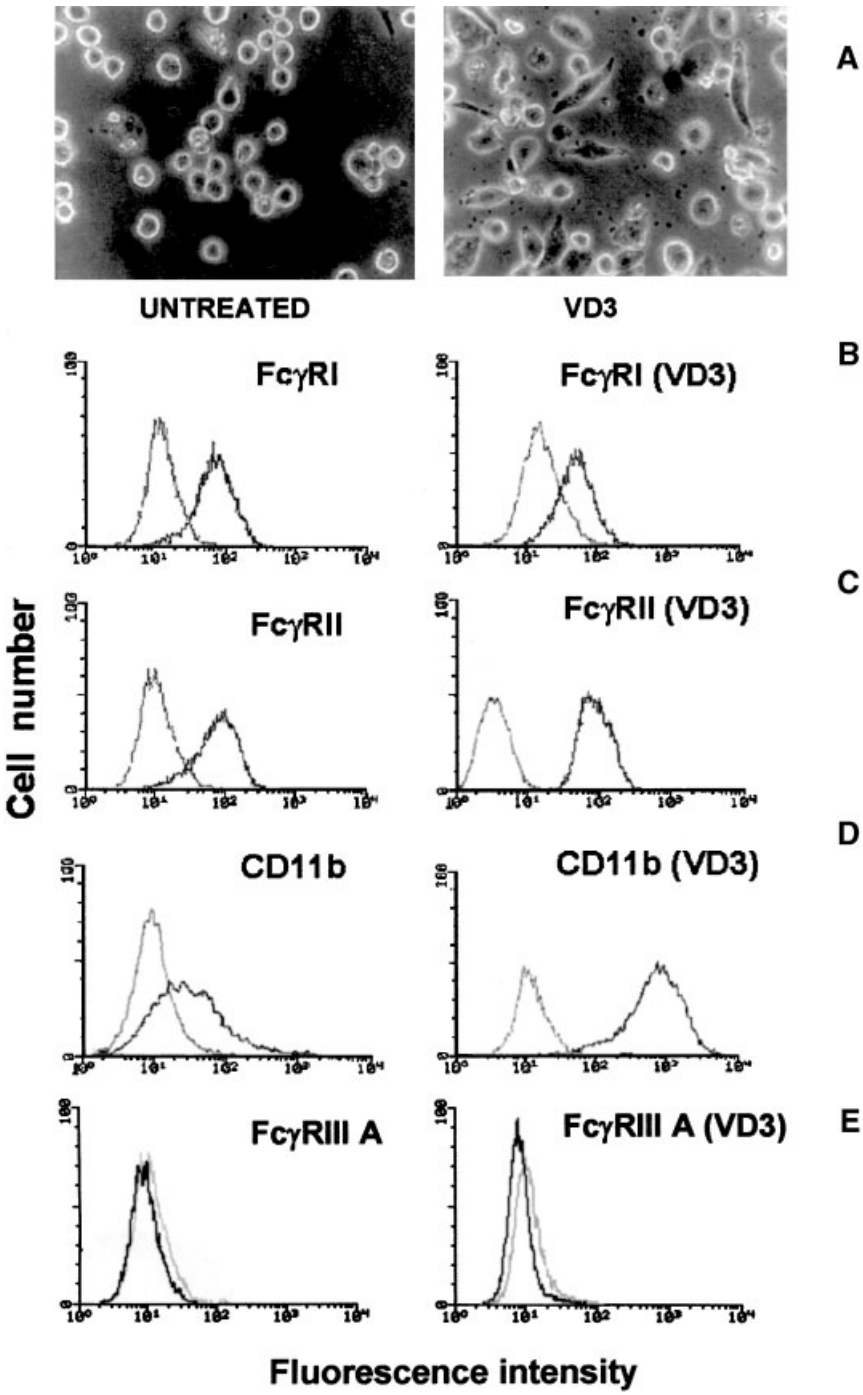

Fig. 1. Effect of VD3-induced differentiation on the morphology and the surface expression of FcγRs and CD11b/CD18 receptors in THP-1 cells. **A**: THP-1 cells (5×10^5/ml) were incubated in plastic culture flasks with or without 100 nM VD3 for 72 h. Microscopic phase-contrast images of the cells in the culture bottles were obtained in an Axiovert 25 (Carl Zeiss) inverted microscope attached to a photographic camera. **B**, **C**, **D**, **E**: Untreated or VD3-treated (100 nM, 72 h) THP-1 cells (0.5×10^6) in 0.5 ml of PBS; 5% FBS; 0.1% NaN_3 were incubated for 60 min at 4°C with 10 μg of mAbs 32.2 (anti-FcγRI), IV.3 (anti-FcγRII), 3G8 (anti-FcγRIIIA) or 2LPM19c (anti-CD11b) or the respective isotype controls (IgG1 for 2LPM19c, 32.2, 3G8 and IgG2b for IV.3). After washing, the cells were stained for 30 min at 4°C with FITC-anti-mouse IgG. The stained cells were washed, fixed in paraformaldehyde and examined by cytofluorometry in a FACScan. **Left column**: undifferentiated THP-1 cells. **Right column**: THP-1 cells treated with VD3. The darker traces are the fluorescence distributions of cells stained with the specific antibodies. Similar results have been observed in numerous experiments performed during the course of the studies reported here.

crosslinking is different, decreasing both the phosphorylation in tyrosine residues and the kinase activity of Syk. To determine if this effect was also observed after crosslinking FcγRI, we performed similar experiments inducing FcγRI crosslinking with anti-FcγRI Fab fragments and secondary antibodies. As expected, in undifferentiated THP-1 cells, FcγRI crosslinking induces Syk phosphorylation and activation (Fig. 5A). VD3 treatment by itself increased

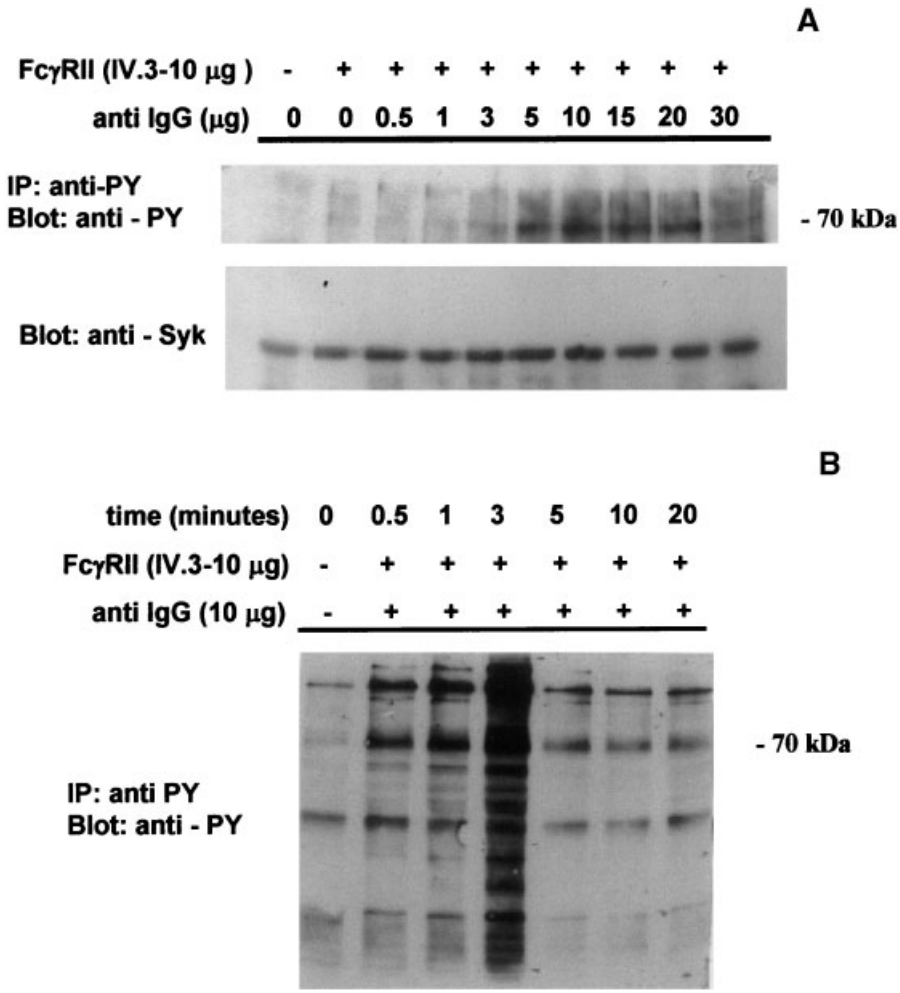

Fig. 2. Tyrosine phosphorylation of Syk induced by FcγRII crosslinking. **A**: THP-1 cells (1×10^7 in 1.0 ml) were incubated with 10 μg of Fab fragments of mAb IV.3 at 4°C for 10 min, centrifuged and resuspended in 1.0 ml RPMI containing the indicated amounts of $F(ab)'_2$ fragments of rabbit anti-mouse IgG for 3 min at 37°C. Stimulation was halted by the addition of 500 μl of ice-cold TBS to each tube, the tubes were centrifuged and the pelleted cells were lysed in lysis buffer. Equivalent amounts of total protein from each lysate were used for imunoprecipitations with anti-Syk antibodies bound to protein A-Sepharose beads to immunoprecipitate Syk. After washing, the immunoprecipitates were boiled in Laemmli sample buffer and resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blot was developed with anti-phosphotyrosine (anti-PY) antibodies as described in Materials and Methods. The same membrane was acid-stripped and reprobed with anti-Syk polyclonal antibodies. **B**: THP-1 cells (1×10^7 in 1.0 ml) were incubated with 10 μg of Fab fragments of mAb IV.3 at 4°C for 10 min. After centrifugation, the cells were resuspended in 1.0 ml RPMI containing 10 μg of $F(ab)'_2$ fragments of rabbit anti-mouse IgG and incubated at 37°C for the indicated times. The cells were lysed and the phosphorylated proteins were immunoprecipitated from equivalent amounts of total protein, and resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blot was developed with anti-phosphotyrosine antibody conjugated to HRP and chemiluminiscent detection. **C**: THP-1 (1 X 10^7 in 1.0 ml) cells were incubated for 10 min at 4°C with RPMI alone or with 10 μg of Fab fragments of mAb IV.3. After centrifugation, the cells were incubated at 37°C for 3 min with 10 μg of $F(ab)'_2$ fragments of anti-mouse IgG in 1.0 ml of RPMI medium. Cells were lysed and Syk was immunoprecipitated with anti-Syk antibodies bound to Protein A-Sepharose beads. Immunoprecipitates were resolved as in A, and the blot was sequentially developed with anti-PY and anti-Syk polyclonal antibodies. **D**: The phosphorylation level of Syk was calculated as the ratio of the densitometric intensities of the anti-PY signal to the anti-Syk signal of the respective bands in three independent experiments as shown in C. Results are the mean ± SEM, n = 3. $^*P < 0.05$ compared with unstimulated cells (**lane 1**).

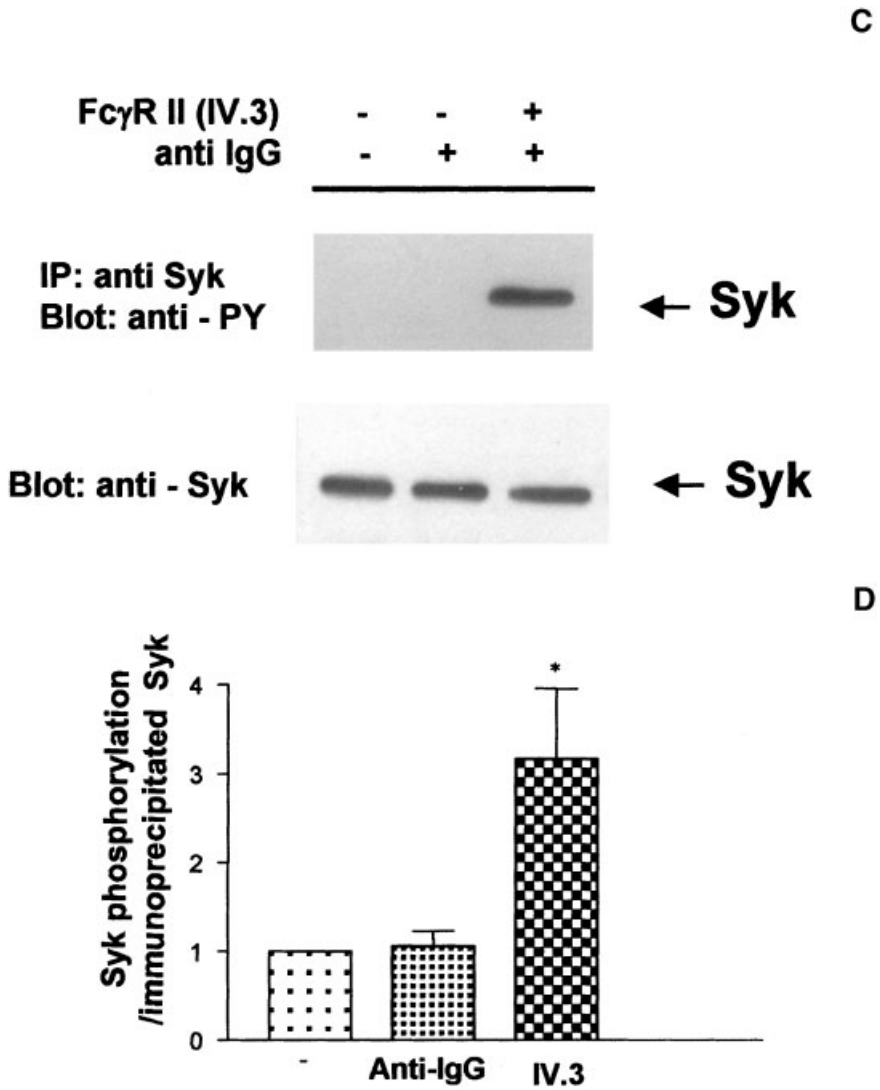

Fig. 2. (*Continued*)

the basal level of Syk phosphorylation, but crosslinking of FcγRI in VD3 treated cells was still able to cause an increase in Syk phosphorylation (Fig. 5B). Thus, VD3 induces an increase in both total level and basal phosphorylation state of Syk, and the cells' response in terms of Syk activation induced by FcγRI or FcγRII crosslinking is differently affected by differentiation.

Effect of VD3 Treatment on FcγR-Mediated Phagocytosis in THP-1 Cells

To determine if VD3 differentiation affects an FcγR-mediated function, we examined the phagocytosis of IgG opsonized erythrocytes by THP-1 cells after VD3 treatment for 0, 24, 48, and 72 h. VD3 treatment for 48 and 72 h decreased THP-1 phagocytosis in a time-dependent manner reaching an inhibition of about 35% after 72 h (Fig. 6). VD3 treatment for 24 h did not result in any significant decrease in phagocytosis (0 vs. 24 h).

Pervanadate Treatment Restores Syk Phosphorylation Induced by FcγRII Crosslinking in VD3-Treated Cells

To determine if the decrease in the phosphorylation state of Syk induced by FcγRII crosslinking in VD3-differentiated cells is mediated by a protein-tyrosine phosphatase activity induced by FcγRII crosslinking, we examined if this effect could be prevented by a general phosphatase inhibitor (sodium pervanadate). VD3 treated cells were incubated in the presence of sodium pervanadate for 5 min before stimulation by FcγRII crosslinking as above. After stimulation of VD3 treated or untreated cells, Syk

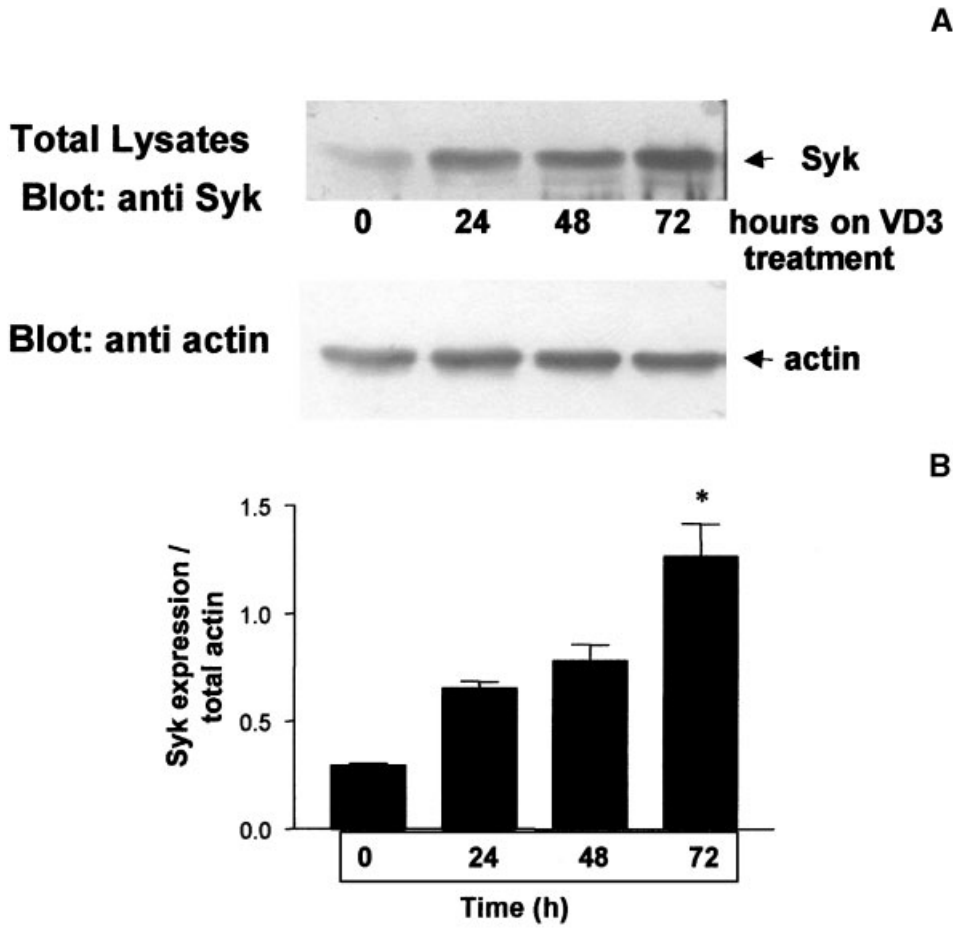

Fig. 3. Effect of VD3 on the expression of Syk in THP-1 cells. **A**: THP-1 cells (5×10^5/ml) were incubated with 100 nM VD3 in RPMI-10% FBS for the indicated times. Cells were washed, lysed in lysis buffer and equivalent amounts of total protein from each lysate were separated by SDS–PAGE. The resolved proteins were transferred to nitrocellulose membranes and sequentially developed with anti-Syk and anti-actin antibodies. **B**: The Syk level was calculated as the ratio of the densitometric intensities of the anti-Syk signal to the anti-actin signal in each lane in three independent experiments. Results are expressed as mean ± SEM, n = 3. $^*P < 0.05$ compared with untreated cells (0 h).

phosphorylation was analyzed by anti-PY/anti-Syk immunoblotting as described above. In VD3-treated cells, pretreatment with the phosphatase inhibitor prevented the previously observed decrease in Syk phosphorylation induced by FcγRII crosslinking (Fig. 7A compare lanes 3 and 5). These results suggest that in VD3-treated cells (but not in undifferentiated THP-1 cells) a protein tyrosine phosphatase is involved in regulation of the Syk phosphorylation level after FcγRII crosslinking.

Interaction Between Syk and SHP-1 is Modulated by VD3

SHP-1 is a protein tyrosine phosphatase, which has been shown to interact directly with ZAP-70 in T cell lines and in heterologous expression systems. Based on this, it has been suggested that SHP-1 is involved in the negative regulation of the catalytic activity of ZAP-70 [Plas et al., 1996]. The physical association of SHP-1 and Syk has also been reported, and it has been shown that Syk is a substrate for SHP-1 [Dustin et al., 1999]. To determine if VD3 treatment modulates SHP-1 expression, we analyzed by immunoblotting the levels of SHP-1 in cells treated with VD3 for different times. The results showed that VD3 treatment did not significantly affect the expression of SHP-1 (Fig. 7B). In order to assess the possible association of SHP-1 with Syk in THP-1 cells and if this association could be modulated by VD3 treatment, we determined by immunoblotting the presence of SHP-1 in anti-Syk immunoprecipitates from VD3 treated and untreated cells before and after FcγRII crosslinking. To obtain a

quantitative assessment of the degree of coimmunoprecipitation of SHP-1 with Syk, the ratio of the densitometric signals of SHP-1 to Syk was calculated. In undifferentiated, non-stimulated THP-1 cells, a certain degree of SHP-1 coimmunoprecipitation with Syk was observed (Fig. 7C, lane 1, ratio = 1.0). Upon FcγRII crosslinking, this association was significantly reduced (Fig. 7C, lane 2). Treatment of the cells with VD3 for 72 h slightly diminished the level of basal association of both molecules (Fig. 7C, lane 3, ratio = 0.80). However, upon FcγR II crosslinking, a significantly higher amount of SHP-1 was coimmunoprecipitated with Syk (Fig. 7C,

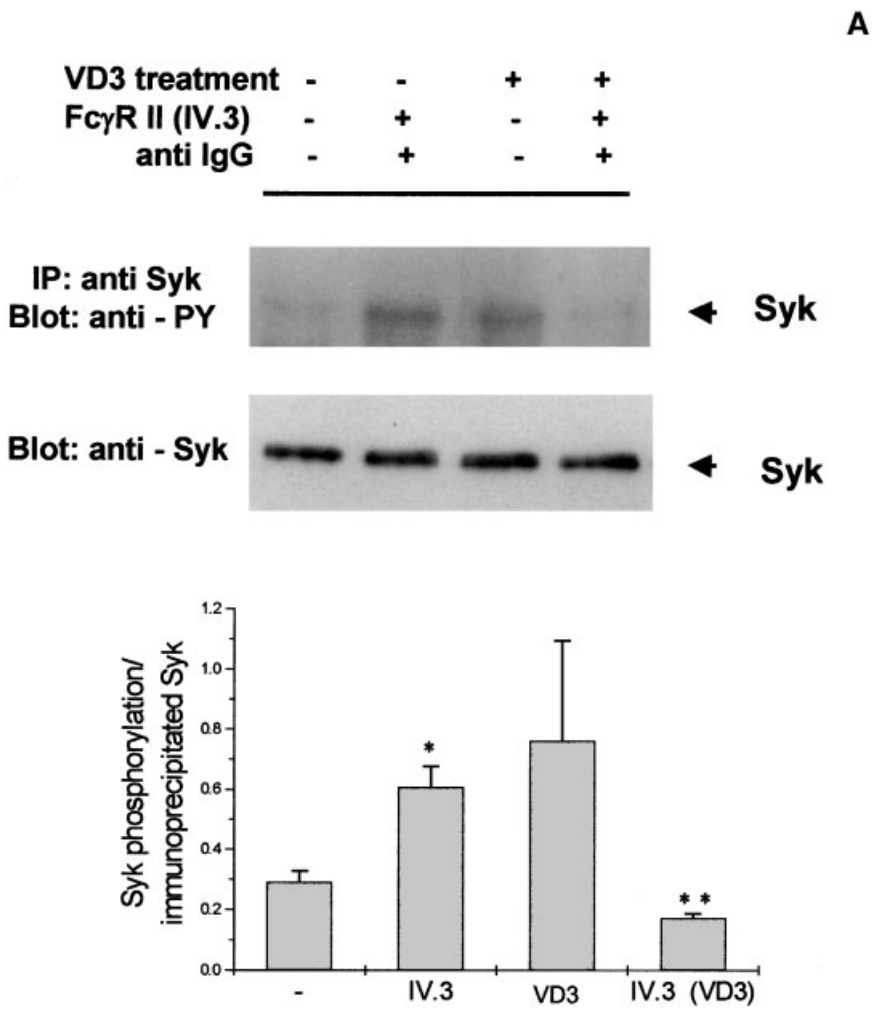

Fig. 4. VD3 treatment of THP-1 cell prevents Syk phosphorylation induced by FcγRII crosslinking. **A**: Tyrosine phosphorylation of Syk in VD3-treated and untreated THP-1 cells. THP-1 cells were left untreated (**lanes 1** and **2**) or were treated with 100 nM of VD3 for 72 h (**lanes 3** and **4**). Treated or untreated THP-1 cells (10^7/ml) were incubated in RPMI medium with 10 μg of Fab fragments of mAb IV.3 (lanes 2 and 4) at 4°C for 10 min, followed by further incubation at 37°C for 3 min, with 10 μg of $F(ab)'_2$ fragments of rabbit anti-mouse IgG. The cells were lysed in lysis buffer and Syk was immunoprecipitated with anti-Syk antibodies bound to Protein A-Sepharose beads from equivalent amounts of cell lysates. The immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blot was developed with anti-phosphotyrosine (anti-PY) antibodies as described in Materials and Methods. The same membrane was acid-stripped and reprobed with anti-Syk polyclonal antibodies. The phosphorylation level of Syk was calculated as the ratio of the densitometric intensities of the anti-PY signal to the anti-Syk signal of the respective bands. The graph shows the results from three independent experiments. Results are expressed as mean ± SEM. n = 3; $*P < 0.05$ compared with unstimulated and untreated cells (lane 1) and $**P < 0.05$ compared with unstimulated and VD3 treated cells (lane 3). **B**: VD3 treatment blocks Syk activation induced by FcγRII crosslinking. Anti-Syk immunoprecipitates from lysates of equivalent number of VD3 treated and untreated cells were obtained from unstimulated or stimulated cells as in A. The immunoprecipitates were resuspended in kinase buffer (with 0.25 Ci of γ-^{32}P-ATP), and used for in vitro kinase assays using 0.25 mg/ml of MBP as substrate. The reaction products were analyzed by SDS–PAGE and autoradiography. The experiment shown is representative of three independent experiments.

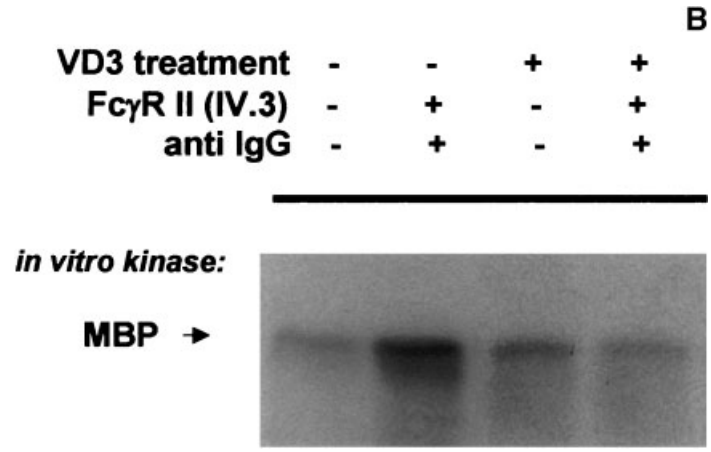

Fig. 4. (*Continued*)

lane 4, ratio = 1.70). The degree of Syk phosphorylation in cells stimulated through FcγRII seems to inversely correlate with the level of Syk-SHP-1 association: in non-treated cells FcγRII crosslinking results in an increase in Syk phosphorylation (and barely detectable association with SHP-1), whereas in VD3 treated cells the level of Syk phosphorylation is low and its association with SHP-1 is highest (Fig. 7D).

Modulation of FcγRII mRNA Levels by VD3 Treatment in THP-1 Cells

The above results suggest that in VD3-treated THP-1 cells, FcγRII crosslinking results in a decrease in the level of Syk phosphorylation, and that this effect could be mediated by modulation of the association of SHP-1 with Syk. A possible mechanism involved in this effect is that VD3 treatment could induce changes in the relative expression of FcγRII isoforms, since one of the FcγRII isoforms, FcγRIIB, is an ITIM-containing receptor which has been shown to recruit SH2-containing protein tyrosine phosphatases (SHP-1 and SHP-2) as well as the phosphatidylinositol 5′-phosphatase SHIP to the vicinity of phospho-ITAMs [Dustin et al., 1999]. Therefore, an increase in the relative levels of FcγRIIB versus FcγRIIA could provide a mechanism to recruit more SHP-1 to the aggregates. To test this possibility, we used a semiquantitative RT-PCR to determine if VD3 modifies the expression of FcγRII isoforms. The primers pairs used to specifically amplify cDNA for FcγRI, FcγRIIa, FcγRIIb$_1$, FcγRIIb$_2$, and FcγRIIc are shown in Table I, and the results of a single experiment as well as the graphs of the combined results of four independent experiments are shown in Figure 8. A PCR product of 439 bp corresponding to the FcγRIa transcript, and two PCR products of 441 and 317 bp, corresponding to FcγRIIa$_1$ and FcγRIIa$_2$ transcripts were obtained using the FcγRI and FcγRIIA specific primers, respectively. No differences in the level of FcγRI and FcγRIIa$_1$ mRNAs were observed after VD3 treatment, while a significant decrease in the level of the 317 bp band (FcγRIIa$_2$) was observed after 48 and 72 h of VD3 treatment (Fig. 8A,B).

Two PCR products were amplified with the use of the FcγRIIb-specific primers: a weak band at 582 bp corresponding to FcγRIIb$_1$ and a fragment of 520 bp corresponding to FcγRIIb$_2$. However, the 582 bp fragment was clearly visible only in one out of four independent experiments and even in that experiment the intensity of this band was very faint. In contrast, the 520 bp fragment (FcγRIIb$_2$) was always clearly detected. This band reproducibly showed a biphasic pattern after VD3 treatment: it decreases after 24 h of treatment and becomes almost undetectable at 48 h. However, after 72 h FcγRIIb$_2$ expression returned to the levels observed in non-treated cells (Fig. 8C).

Analysis of FcγRIIc mRNAs by RT-PCR showed a band corresponding to 377 bp. No significant changes in the level of expression of this band were detected after VD3 treatment (Fig. 8D). The expression of β-actin was not modified by VD3 treatment (Fig. 8E). The RT-PCR for the actin mRNA was included as an xinternal control and to normalize the levels of FcγRs mRNAs among the different experiments.

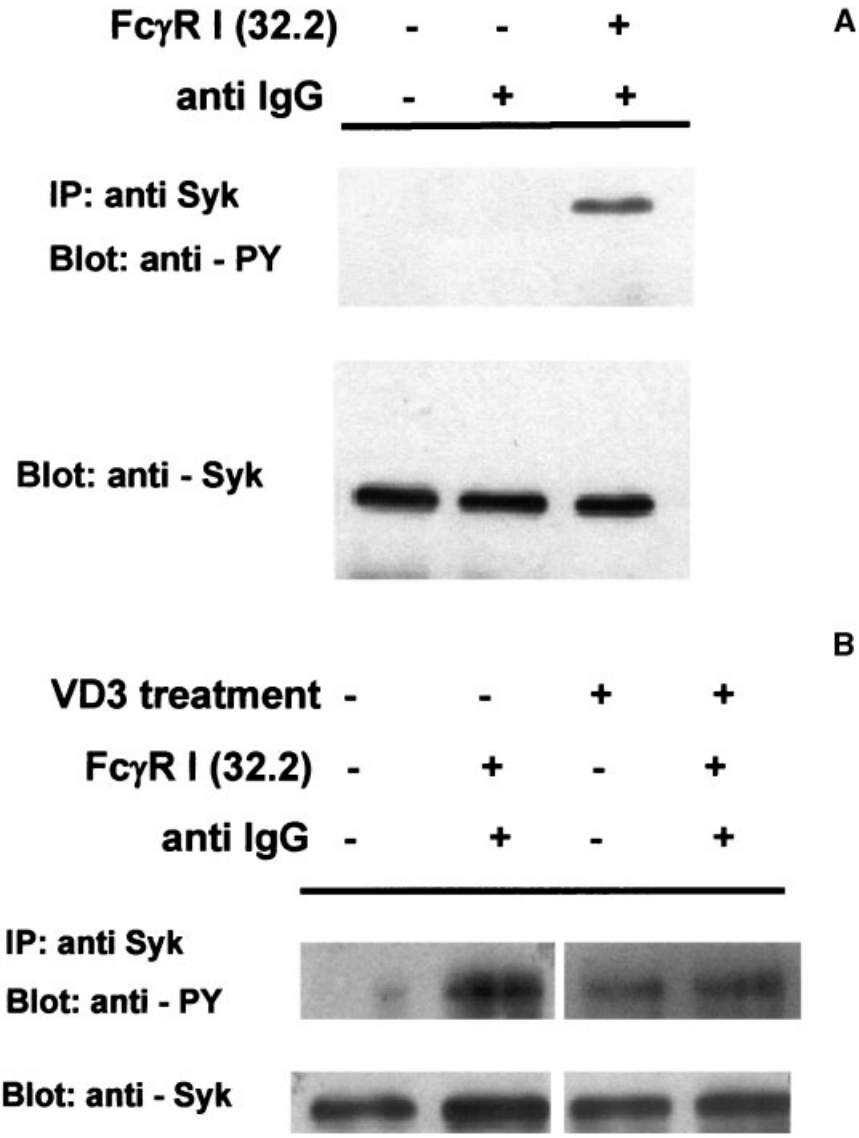

Fig. 5. VD3 treatment of THP-1 does not block Syk phosphorylation induced by FcγRI crosslinking. **A**: THP-1 cells (1×10^7 in 1.0 ml) were incubated in RPMI medium alone or with 10 μg of Fab fragments of mAb 32.2, at 4°C for 10 min, followed by further incubation at 37°C for 3 min, with 10 μg of $F(ab)'_2$ fragments of rabbit anti-mouse IgG. The cells were lysed in lysis buffer and equivalent amounts of total protein from each lysate were used to immunoprecipitate Syk. The immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blot was developed with anti-phosphotyrosine (anti-PY) antibodies as described in Materials and Methods. The same membrane was acid-stripped and reprobed with anti-Syk polyclonal antibodies. **B**: THP-1 cells were left untreated (**lanes 1** and **2**) or were treated with 100 nM of VD3 for 72 h (**lanes 3** and **4**). Treated or untreated THP-1 cells (10^7/ml) were stimulated as in A. The cells were lysed in lysis buffer and Syk was immunoprecipitated with anti-Syk antibodies bound to Protein A-Sepharose beads from equivalent amounts of cell lysates. The immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blot was developed with anti-phosphotyrosine (anti-PY) antibodies as described in Materials and Methods. The same membrane was acid-stripped and reprobed with anti-Syk polyclonal antibodies.

DISCUSSION

Receptors for the Fc portion of IgG are expressed on the surface of almost all hematopoietic cells. Binding of antigen-antibody complexes or IgG-opsonized particles to these receptors triggers a variety of effector responses. These include phagocytosis, production of cytokines and chemokines, release of cytotoxic and microbicidal molecules, and changes in the expression of cell-surface proteins involved in cell–cell adhesion and antigen presentation [Ravetch and Kinet, 1991]. In this way, FcγRs allow the humoral and cellular aspects of immunity to

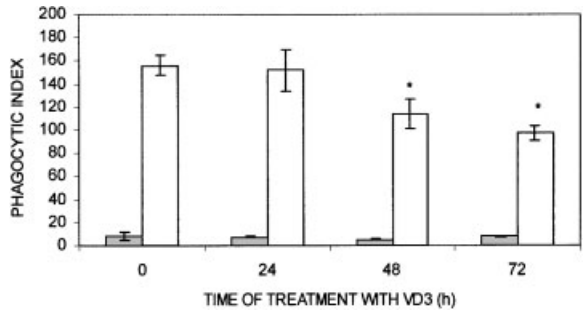

Fig. 6. VD3 treatment decrease FcγR-mediated phagocytosis in THP-1 cells. Sheep RBC were opsonized with anti-DNP IgG as described in Materials and Methods. For the phagocytosis assay, 320 μl of THP-1 cells suspension (1×10^6/ml) were incubated with 60 μl of a 2% suspension of opsonized (empty bars) or non-opsonized (gray bars) RBC for 2 h at 37°C in a 5% carbon dioxide humidified incubator. The cells were then washed three times with PBS to remove unbound RBCs. Non-internalized RBCs were lysed with 0.2% PBS for 30 s. RBC ingestion by THP-1 cells was examined by light microscopy by an observer who was blind to treatment conditions. All assays were performed in triplicate. The results are expressed as the phagocytic index (number of ingested RBC by 100 cells). Results are expressed as mean ± SD. $n = 3$, $^*P < 0.05$ compared with the untreated cells (0 h).

communicate and cooperate in expanding, sustaining, and regulating immune responses.

Numerous studies have identified a variety of molecular participants and biochemical events involved in signal transduction through FcγRs [Daeron, 1997; Ravetch and Bolland, 2001]. Less attention has been given to study how the biochemical pathways initiated by individual types of FcγRs can be affected by the differentiation/activation state of the cell expressing the FcγR. The aim of this study was to determine if the biochemical events initiated by aggregating FcγRII on monocytic cells are affected by the differentiation state of the cell.

Syk is known to play a central role in the signaling pathways activated by FcγRs [Agarwal et al., 1993; Kiener et al., 1993; Pan et al., 1999]. Once activated, Syk can stimulate various biochemical pathways involved in the cell's response. Because of its central role, we decided to study the effect of cell differentiation on signal transduction by FcγRII by focusing on the level of phosphorylation and activation of Syk after aggregation of FcγRII in a monocytic human cell line that can be differentiated in vitro. The metabolite 1α,25-dihidroxy-vitamin D3 (VD3) has been shown to promote differentiation of monocytic cell lines towards a macrophage-like phenotype [Choudhuri et al., 1990; Kreutz and Andreesen, 1990]. Treatment of THP-1 cells for 72 h with VD3 induces several differentiation-related changes, such as an increase in CD14 and CD11b/CD18 (CR3) expression, and an increased adherence to plastic surfaces. We showed here that in vitro treatment of THP-1 cells with VD3 also induces a time-dependent increase in Syk levels (Fig. 3A). The basal (unstimulated) level of tyrosine phosphorylation of Syk was also increased after VD3 treatment. This results from a two to three fold increase in the amount of immunoreactive phosphotyrosine on Syk, as determined by comparing the ratio of the anti-phosphotyrosine signal with the anti-Syk signals in Syk immunoprecipitated from cells before and after treatment with VD3 for 72 h (Fig. 4A). Thus, during VD3 induced differentiation of THP-1 cells, along with an increase in the total amount of Syk, there is a definite increase in the basal phosphorylation of Syk.

The increase in the levels of tyrosine phosphorylation induced by VD3 correlate with an increase in the catalytic activity of Syk against an exogenous substrate in in vitro kinase assays (Fig. 4B, lane 3). It is interesting that a similar increase in tyrosine phosphorylation and catalytic activity of Syk has also been reported during differentiation of HL-60 promyelocitic cells into granulocytes [Qin and Yamamura, 1997]. Syk has different tyrosines which can be phosphorylated/dephosphorylated and some of these have been implicated in regulation of its catalytic activity. It is also known that VD3 activates a variety of proteins with kinase activity, such as protein kinase C, Raf, and mitogen-activated protein (MAP) kinases [Kharbanda et al., 1994; Marcinkowaska et al., 1997; Gniadecki, 1998], involved in the differentiation process and that can potentially phosphorylate Syk tyrosine residues. At this time, the mechanism and functional significance of the increase in tyrosine phosphorylation and activity of Syk induced by differentiation (by VD3 in THP-1 cells, by retinoic acid in HL-60 cells) is unknown, and also if other differentiation inducing agents have similar effect.

As has been reported, in undifferentiated THP-1 cells, FcγRII crosslinking induced a dose-dependent increase in the level of Syk tyrosine phosphorylation. Surprisingly, in VD3-treated cells FcγRII crosslinking not only failed to induce an increase in Syk phosphorylation, but it actually induced a decrease in Syk phosphorylation to levels lower than those observed in unstimulated cells. As a read out

assay to evaluate the effect of the decrease in Syk phosphorylation on an FcγR mediated signal, we determined if VD3 treatment would inhibit FcγR-mediated phagocytosis of IgG-coated SRBC. The phagocytosis of IgG-coated RBC decreased only about 35% with VD3 treatment (Fig. 6). However, it should be noted that during IgG-mediated phagocytosis Syk activation induced by FcγRI is still taking place. As we show in Figure 5, Syk phosphorylation induced by FcγRI is not affected by the VD3 treatment, and thus can partially compensate

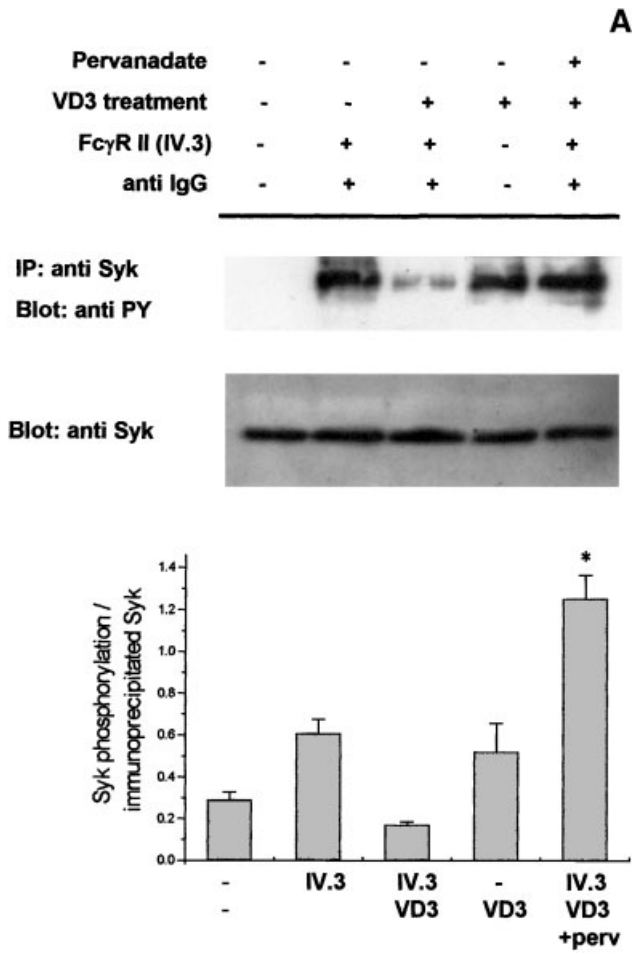

Fig. 7. Syk phosphorylation level after FcγRII crosslinking could be modulated by a protein tyrosine phosphatase. **A**: THP-1 cells were left untreated (**lanes 1** and **2**) or were treated with 100 nM of VD3 for 72 h (**lanes 3–5**). Just before stimulation a sample of VD3 treated cells (lane 5) was incubated with 100 μM sodium pervanadate for 5 min at 37°C. The cells were then stimulated through FcγRII or left unstimulated as described in legend to Figure 2C. Syk was immunoprecipitated from equivalent amounts of cell lysates and the immunoprecipitates were separated on SDS–PAGE and transferred to nitrocellulose membranes. The blots were sequentially probed with anti-PY and anti-Syk antibodies. The graph show the results of three independent experiments expressed as the mean ± SEM. n = 3, $^{*}P < 0.05$ compared with FcγRII stimulated and VD3 treated cell (lane 3). **B**: Effect of VD3 on expression of SHP-1. THP-1 cells (5×10^5/ml) were incubated with 100 nM VD3 for the indicated times. Lysates were prepared and equivalent amounts of total protein from each lysate were separated by SDS–PAGE. The resolved proteins were transferred to nitrocellulose membranes and sequentially developed with anti-SHP-1 and anti-actin antibodies. The graph shows the average of three independent experiments. **C**: Coimmunoprecipitation of SHP-1 and Syk. VD3-treated (lanes 3 and 4) or untreated (lanes 1 and 2) THP-1 cells were stimulated through FcγRII as described in legend to Figure 2C. Anti-Syk immunoprecipitates were prepared from equivalent amounts of cell lysates. The immunoprecipitated proteins were separated on SDS–PAGE, transferred to nitrocellulose and probed with anti-Syk and anti-SHP-1 antibodies. The graph shows the mean ± SEM of three independent experiments; $^{*}P < 0.05$ compared with untreated and unstimulated cells (lane 1) and $^{**}P < 0.05$ compared with stimulated, untreated cells (lane 2). **D**: Syk-SHP-1 association inversely correlates with the level of Syk phosphorylation. VD3-treated (lanes 3) or untreated (lanes 1 and 2) THP-1 cells were stimulated through FcγRII as described in legend to Figure 2. Syk was immunoprecipitated from the cell lysates and the immunoprecipitates were resolved by SDS–PAGE. The resolved proteins were transferred to nitrocellulose membranes which were sequentially probed with anti-PY, anti-Syk, and anti-SHP-1 antibodies. Results are representative of three independent experiments.

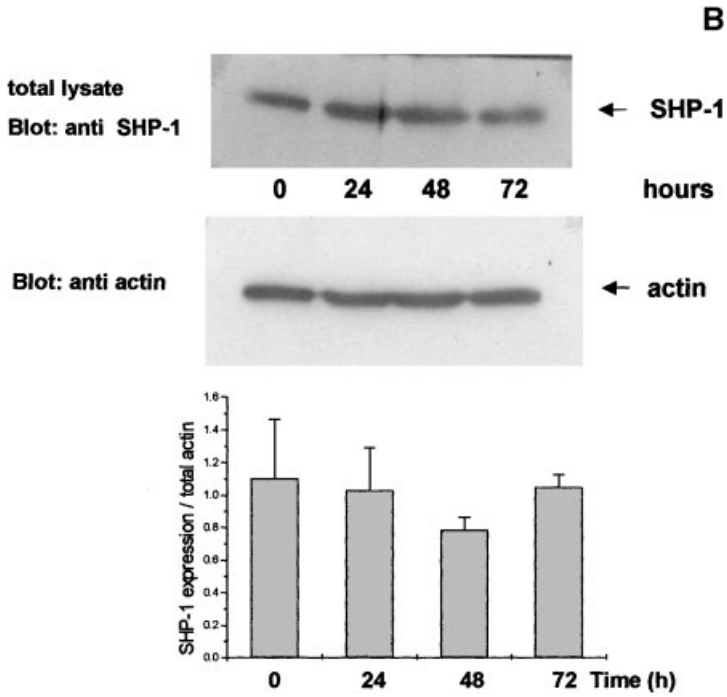

Fig. 7. (*Continued*)

the deficient Syk activation induced by FcγRII in cells differentiated by VD3.

The possibility that this decrease in Syk phosphorylation is mediated by the FcγRII crosslinking-induced activation of a protein phosphatase was suggested by the fact that this effect was eliminated by previous treatment of the cells with sodium pervanadate, a general phosphatase inhibitor. This suggests that a phosphatase activity, which is affected by FcγRII crosslinking, is involved in regulating the state of Syk phosphorylation. Several lines of evidence have pointed to the protein tyrosine phosphatase SHP-1 as a negative regulator of signaling through ITAM-containing immunoreceptors by acting on tyrosine kinases of the Syk/ZAP-70 family. SHP-1 has been implicated in the negative regulation of the activity of ZAP-70 in T lymphocytes [Plas et al., 1996]. It has also been shown that in B cells there is a physical association of SHP-1 with Syk and that Syk is a substrate for SHP-1 [Dustin et al., 1999]. In *me/me* mice, SHP-1 was proposed to be a critical molecule used by FcγRIIB for down-regulation of BCR signaling [Dustin et al., 1999]. In vitro studies have shown that SHP-1 can bind to the ITIM motif of FcγRIIB [Leosurne et al., 2001]. Our results showed that in THP-1 cells, SHP-1 can be shown to coimmunoprecipitate with Syk, and that the degree of coimmunoprecipitation is affected by the differentiation state of the cell and by FcγRII crosslinking. It is important to note that SHP-1 expression level was not altered by treatment with VD3 (Fig. 7B). In resting, undifferentiated cells, a small but definite amount of SHP-1 is reproducibly found in anti-Syk immunoprecipitates. Since in these conditions Syk is not phosphorylated to detectable levels, the molecular basis for this constitutive association in THP-1 cells is unknown. It is possible that SHP-1 interacts with Syk by a mechanism independent of the binding of SH2 domains of SHP-1 to phosphorylated tyrosine residues in Syk. In this respect, it has been reported that the functional interaction between SHP-1 and JAK2 is independent of tyrosine phosphorylation of JAK2, and does not require the functional SH2 domains of SHP-1 [Jiao et al., 1996]. In U937 cells the constitutive association of SHP-1 and Lyn has been reported to be dependent on Lyn SH3 domain [Yoshida et al., 1999; Somani et al., 2001].

The constitutive association of Syk and SHP-1 in undifferentiated cells is lost upon FcγRII crosslinking, concomitant with the increase in Syk tyrosine phosphorylation. These results are in contrast with reports in B cells showing that BCR engagement and subsequent Syk tyrosine phosphorylation does not affect the association of Syk and SHP-1 [Dustin et al., 1999].

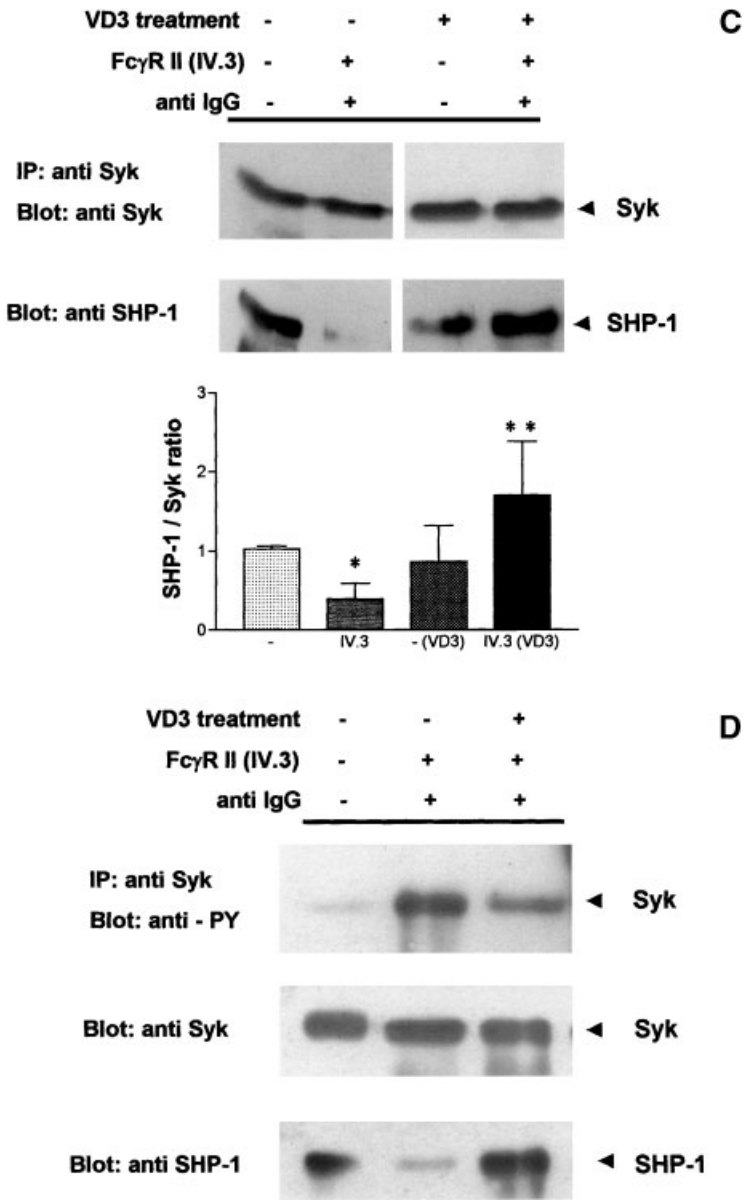

Fig. 7. (*Continued*)

Differentiation for 72 h induced by VD3 slightly diminished the level of basal association of Syk and SHP-1 (Fig. 7C). In contrast to undifferentiated cells, FcγRII crosslinking induced a significant increase in the amount of SHP-1 that coimmunoprecipitates with Syk and a significant decrease in the level of Syk phosphorylation (Fig. 7C,D). Taken together, these observations suggest that in VD3 treated monocytic cells, Syk could be negatively regulated by SHP-1 mediated dephosphorylation after FcγRII crosslinking.

A possible mechanism by which differentiation by VD3 might modify the physical and functional interaction between Syk and SHP-1 after FcγRII crosslinking with respect to undifferentiated cells, is that VD3 treatment alters the relative expression of FcγRII isoforms, inducing an increase in the expression of FcγRIIB, thus promoting SHP-1 recruitment to the FcγRII aggregates by binding to the FcγRIIB ITIMs. SHP-1 has been shown to bind to FcγRIIB phosphorylated ITIMs [D'Ambrosio et al., 1996; Sato and Ochi, 1998], and also to associate with FcγRIIB when it is co-aggregated with the type I FcεR in bone marrow derived mast cells [Fong et al., 1996]. We observed that VD3 induced a transient decrease in FcγRIIb expression, but

after 72 h of VD3 treatment expression of $Fc\gamma RIIb_1$ and $Fc\gamma RIIb_2$ returned to the level observed in non-differentiated cells (Fig. 8C). Although this does not rule out the possibility that in VD3-differentiated cells SHP-1 gains access to the vicinity of Syk due to its recruitment to the receptor aggregates by interacting with FcγRIIB ITIMs, it does indicate that the differences in Syk-SHP-1 interactions observed between differentiated and undifferentiated cells are regulated by factors other than differentiation-induced changes in expression of FcγRII isoforms. Another possibility is that although the expression of FcγRIIB does not change in VD3 differentiated cells, the level of phosphorylation of its ITIM motif is higher, enhancing its ability to recruit SHP-1 [Leosurne et al., 2001]. Differentiation-regulated changes in SHP-1 activation have been previously reported in myeloid cells [Uesugi et al., 1999, 2000].

Our findings are consistent with a scheme in which, in undifferentiated resting cells, the basal phosphorylation level of Syk is regulated by SHP-1, which is constitutively associated to Syk in a phosphorylation independent way. FcγRII crosslinking induces dissociation of SHP-1-Syk complexes, and causes Syk to be recruited to the phosphorylated FcγRII ITAMs and activated. Differentiation by VD3 modifies the interplay between Syk and SHP-1 such that the basal phosphorylation level of Syk increases, and FcγRII crosslinking promotes both

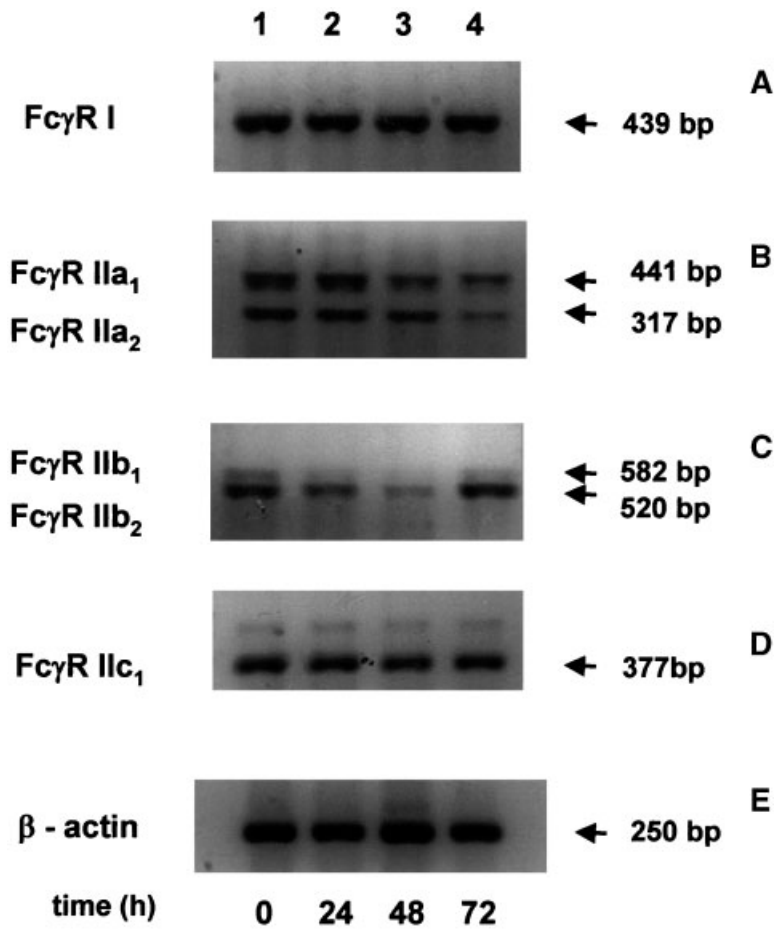

Fig. 8. Effect of VD3 treatment of THP-1 cells on the expression of transcripts for FcγR isoforms. RNA was isolated from THP-1 cells treated for 0, 24, 48, and 72 h with VD3 and was reverse transcribed and PCR amplified with the FcγRII A, B, and C specific primer pairs indicated in Table I. After the PCR reaction, the products were analyzed on a 2% agarose gel containing EtBr. **A**: FcγR I; (**B**) FcγR II a_1, a_2 isoforms; (**C**) FcγR II b_1, b_2 isoforms; (**D**) FcγR II c_1 isoform; and (**E**) β-actin. Specific bands with the expected sizes for each primer pair were detected in each panel. PCR products were quantified by densitometric analysis and their expression is presented as relative to the data of β-actin mRNA densitometric values. The graphs (**F**) show the relative levels of FcγR isoform-specific transcripts after 0, 24, 48, and 72 h of VD3 treatment obtained from four independent experiments (mean ± SEM of four independent experiments; $^*P < 0.05$ compared with untreated cells, 0 h).

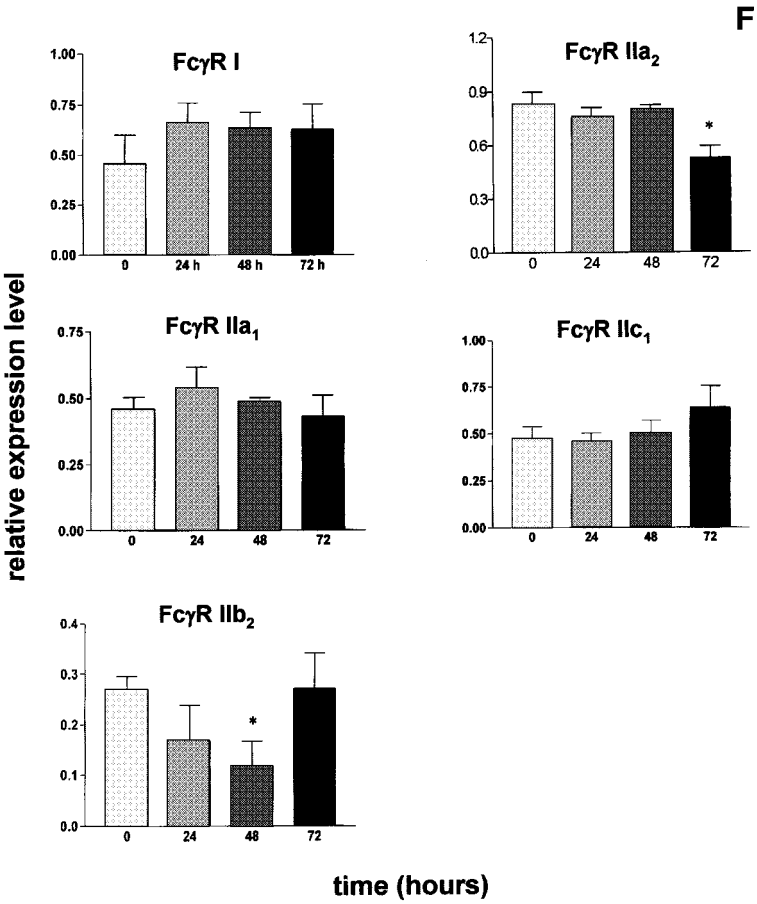

Fig. 8. (*Continued*)

SHP-1 activation and a decrease in the level of Syk phosphorylation and activity. Surely, other molecules might be participating in the differentiation related changes in regulation of Syk activity; further studies are necessary to dissect the mechanisms involved in this effect.

Analysis of the effect of VD3 on the expression of FcγRII isoforms showed a significant decrease in $Fc\gamma RIIa_2$ mRNA after 72 h of treatment. The $Fc\gamma RIIa_2$ is a soluble form of FcγRIIa lacking the transmembrane exon [Rappaport et al., 1993]. Transcripts encoding this form of FcγRIIa were identified in megakariocyte-like human cell lines and platelets [Rappaport et al., 1993], and in Langerhans cells [Astier et al., 1994]. Our results show that this form of FcγRIIa is also produced by the monocytic cell line THP-1, and that its expression is modulated by differentiation with VD3. Although soluble $Fc\gamma RIIa_2$ might be important in vivo in modulating interaction of immune complexes with FcγRs, its expression in our experimental system could not influence our results as soluble FcγR forms would be eliminated by washing before the stimulation.

Our findings have established that the effect of FcγRII crosslinking on Syk tyrosine phosphorylation and activation differs between undifferentiated and VD3-differentiated THP-1 cells. This effect is not mediated by changes in the expression of FcγR isoforms. Other studies have shown a switch in FcγRI signaling pathways upon monocyte differentiation by IFN-γ, mediated by a switch in the accessory molecule recruited by FcγRI, which lacks its own intrinsic signaling motif [Melendez et al., 1998]. Together, these findings illustrate that the biochemical pathways resulting from crosslinking of a particular FcγR do not depend solely on the particular FcγR isoform that is aggregated, or on the cell type, but that they are also highly dependent on the differentiation state of the cell, which in vivo is subjected to the action of a variety of stimuli. Understanding the mechanism by which the cell can modulate the transduction pathways induced by the large family of FcγRs could help to

understand the biological significance of the heterogeneity found in this family of receptors that respond to the same ligand, IgG immune complexes or IgG opsonized particles.

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