Serine/threonine phosphorylation of the γ -subunit after activation of the high-affinity Fc receptor for immunoglobulin G

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In this report we show that interferon γ treatment of U937 cells induces increased expression of the γ -subunit of the high-affinity Fc receptor for IgG (Fc γ RI). Interferon treatment results in a 10-fold increased expression of the γ -subunit and induces expression of a phosphorylated form (γ^1). The increased expression of the γ -subunit correlates with its ability to transmit a signal via Fc γ R, as measured by activation of the respiratory burst using insoluble immune complexes. During Fc γ R activation, a mobility shift occurs in the phosphorylated form of this γ^1 -subunit. Phospho-amino acid analysis demonstrates that this γ^1 subunit is threonine phosphorylated in resting differentiated U937 cells and becomes predominantly serine phosphorylated on Fc receptor activation. The mobility shift in the γ -subunit can be induced by treating

U937 cells with phorbol 12-myristate 13-acetate or by monoclonal antibody cross-linking of Fc γ RI. Hence the γ -subunit is serine phosphorylated in response to Fc γ RI and protein kinase C activation. Therefore the γ -subunit, initially described as a subunit of Fc ϵ RI, now appears to be involved in signal transduction via Fc γ RI. The data also suggest that the γ -subunit, in contrast with the ζ -subunit of the T-cell receptor–CD3 complex, is a substrate for serine/threonine kinase(s) in the cell. The serine phosphorylation of the γ -subunit suggests a divergence of structure and function between the γ -subunit and its homologue, the ζ -subunit of the T-cell receptor. Phosphorylation of the γ -subunit on serine may play some regulatory role in Fc γ RI signal transduction in myeloid cells.

INTRODUCTION

The ζ -subunit of the T-cell receptor (TCR)–CD3 complex and the γ -subunit of the high-affinity Fc receptor for IgE (Fc ϵ RI) are tyrosine phosphorylated after activation of TCR and Fc ϵ RI respectively [1,2]. The phosphorylated tyrosine residues in the ζ - and γ -subunits are contained within an 18-residue repetitive signalling motif (NQLYXXLNXXXXXXXXXXL) [3] (see Figure 1). Other members of the immunoglobulin gene superfamily have been shown to contain this repetitive signalling motif also referred to as the antigen receptor homology 1 (ARH1) domain [4]. These include the δ - and γ -subunits of the CD3 complex, the β -subunit of Fc ϵ RI and the mb1(Ig α) and B29(Ig β) proteins associated with the surface immunoglobulin receptor [4–6].

Our work has focused on the γ -subunit which was originally described as a part of the tetrameric Fc receptor for IgE (Fc ϵ RI) in mast cells [7]. In natural killer cells it is a part of Fc γ RIII [8], and, in mouse macrophages, it is associated with another Fc receptor, Fc γ RIIA [8]. It has recently been demonstrated that the γ -subunit is non-covalently associated with Fc γ RI [9,10], suggesting that it may be involved in signal relay via this receptor. Figure 1 shows an alignment of the amino acid sequence of a portion of the cytoplasmic domain of the human ζ - and γ -subunits, including their ARH1 domains [1,7,8,11]. The γ - and ζ -subunits are members of a new family of signal-relay molecules [7,11,12,13]. They are encoded by two separate genes located on mouse and human chromosomes number 1 [7]. A genomic analysis of the exon/intron structures of these two related proteins suggests that they arose via gene duplication [7]. A third member of this family, the η -subunit, is an alternatively spliced form of the ζ -subunit [14]. As shown in Figure 1, an alignment

of a portion of the cytoplasmic domain of the γ - and ζ -subunits based on similarities in the genomic organization of exons 7 and 8 of the ζ -subunit and 4 and 5 of the γ -subunit shows a considerable degree of conservation [13]. The human ζ - and γ -subunits show 44% sequence similarity in the cytoplasmic domain corresponding to exons 4 and 5 of the γ -subunit shown in Figure 1, and they both contain the ARH1 motif (Figure 1).

U937 cells differentiated in recombinant interferon γ (IFN), designated U937IF cells, have a mature macrophage phenotype and serve as a useful model for studying macrophage function and signal relay [15]. In the present report, we show that IFN induces the expression of the native γ -subunit in U937 cells, and this expression correlates with the acquisition of signal transduction through Fc γ R. IFN induces the expression of a phosphorylated form of the γ -subunit, and this γ^1 form (in contrast with the ζ -subunit of the TCR) is predominantly phosphorylated on serine and to a lesser extent on tyrosine in response to Fc γ R activation. Phorbol 12-myristate 13-acetate (PMA) and Fc γ RI stimulation of U937IF cells results in an identical mobility shift, suggesting that the γ^1 -subunit is a substrate for a serine/threonine kinase in the cell. Our data suggest that the γ -subunit, unlike other members of the ARH1 family, is a substrate for serine phosphorylation in the cell and that it is involved in signal transduction via Fc γ RI.

MATERIALS AND METHODS

Cells

U937 and P815 cell lines were obtained from Dr. Irwin Bernstein at the Fred Hutchinson Cancer Center and cultured in RPMI 1640 plus 10% fetal bovine serum. U937IF cells were prepared

Abbreviations used: FcR, Fc receptor; IC, insoluble immune complexes; TCR, T-cell receptor; HBSS, Hanks balanced-salt solution; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Fc γ R, Fc receptor for IgG; Fc γ RI, high-affinity Fc receptor for IgG; Fc ϵ RI, Fc receptor for IgE; IFN, interferon γ ; mAb, monoclonal antibody; ARH1, antigen receptor homology 1.

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The anti-peptide antibody (5927.2) was used to immunoprecipitate the γ -subunit from lysates of resting or stimulated U937 or U937IF cells (4×10^6). Cell lysates were prepared in a lysis buffer containing 0.1 % (w/v) SDS, 10 mM Hepes, pH 7.4, 0.1 M NaCl, 1 mM phenylmethanesulphonyl fluoride, 20 μ g/ml aprotinin, 2 mM EDTA, 50 mM NaF, 0.1 % 2-mercaptoethanol, 10 μ M phenylarsine oxide and 100 μ M vanadate (RIPA IPB). The lysates were cleared by centrifugation at 15000 g for 45 min at 4 °C. The γ -subunit was precipitated by the addition of 5 μ l of 5927.2 or 5932.2 antiserum to clarified cell lysates. After 2 h at 0 °C, 50 μ l of a 10 % solution of formalin-fixed *Staphylococcus aureus* was added, and the mixture incubated for an additional hour at 0 °C. The resultant ICs were washed several times in RIPA IPB. Then 25 μ l of 1 $\times$ Laemmli sample buffer [19] was added, and the samples were heated at 98 °C for 5 min.

Electrophoresis and immunoblotting

Immunoprecipitates and whole-cell lysates were resolved on 20% acrylamide/0.193% bisacrylamide gels by SDS/PAGE as described [21]. Proteins were transferred to nitrocellulose filters (1 mA/h per cm²) using a dry transfer system (Ellard Inc., Seattle, WA, U.S.A.) as described previously [21]. The blot was incubated with blocking solution (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% BSA, 1% ovalbumin) for 1 h at room temperature and then with specific anti- γ -subunit serum (5927.2) (1:2000 dilution in blocking solution) for 2 h at room temperature with continuous agitation. The secondary antibody was an alkaline phosphatase-conjugated goat anti-(rabbit IgG antibody) diluted 1:3000 in blocking solution. Control immunoblots probed with secondary antibody alone showed no detectable γ -subunit signal. For the peptide-block experiments, the primary antibody (diluted 1:2000 in block) was preincubated with the peptide at a concentration of 10 μ g/ml for 30 min before being used in an anti- γ -subunit immunoblot. The IC-detection system was 0.33 mg/ml *p*-Nitro Blue Tetrazolium chloride and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) in 100 mM Tris/HCl, pH 9.5, containing 100 mM NaCl and 50 mM MgCl₂.

Respiratory burst

The generation of O₂⁻ by U937 and U937IF cells was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* [22] at 550 nm in a microtitre plate reader (Molecular Devices Inc., Menlo Park, CA, U.S.A.), using air-oxidized and dithionite-reduced cytochrome *c* as standards. Cells were preincubated for 10 min at 37°C in HBSS in the wells of a 96-well microtitre plate. The final reaction mixture contained 2 $\times$ 10⁶ U937 or U937IF cells and 80 μ M ferricytochrome *c* in 250 μ l of HBSS. One-half of the wells received superoxide dismutase (25 μ g/ml). After the addition of IC or PMA (1 μ g/ml), the plates were incubated at 37°C and agitated. Serial spectrophotometric determinations were recorded to construct a kinetic curve for the production of O₂⁻. Maximum reduction of ferricytochrome *c* (25 nmol) was achieved by adding 5 μ l of freshly prepared sodium dithionite. O₂⁻ production is expressed as nmol of superoxide dismutase-inhibitable cytochrome *c* reduction per 2 $\times$ 10⁶ cells. For comparison of U937IF-cell respiratory-burst activity and biochemical changes in the γ -subunit, separate parallel samples of the U937 and U937IF cells were stimulated with the same agonists.

Metabolic labelling experiments

U937IF cells were metabolically labelled with [³²P]_i (1 mCi/ml for 4 h at 37°C) in labelling medium [Dulbecco's modified Eagle's medium containing 10% of the normal phosphate concentration, 10 mM Hepes, pH 7.2, and 5% (v/v) dialysed fetal calf serum (Gibco/BRL)] Then 4 $\times$ 10⁶ cells were resuspended in 1 ml of labelling medium and incubated at 37°C for 4 h. These cells were then stimulated by adding IC (50 μ g) at room temperature with constant agitation for various lengths of time. The cells were rapidly cooled by the addition of a 10-fold volume of cold HBSS, and collected by centrifugation at 500 *g*. They were then transferred to a 1.5 ml Microfuge tube and lysed in RIPA IPB. Immunoprecipitation and resolution of the γ -subunit protein was as described above. Anti- γ -subunit Western-blot analysis together with autoradiography were used to colocalize the ³²P-labelled γ -subunit.

Figure 2 Characterization of anti- γ -subunit sera

(a)–(d) represent anti- γ -subunit immunoblots (using the 5927.2 antiserum) performed on proteins resolved by SDS/PAGE (20% gels) under reducing conditions except for (c) (lane 3) which is a non-reducing gel. Two anti-peptide sera were generated against the peptide shown in Figure 1, representing the extreme C-terminus of the γ -subunit (designated 5927.2 and 5932.2). Lanes 1–4 represent immunoprecipitation of U937-cell lysates (day 4 of IFN differentiation) with preimmune antisera (lanes 1 and 3) or immune antisera, designated 5927.2 or 5932.2 (lanes 2 and 4 respectively). Lanes 5, 6, 7 and 8 are U937-cell lysates from cells differentiated for 0, 2, 3 or 4 days in IFN respectively. Lanes 9 and 10 are whole-cell lysates of a Jurkat T-cell line and Raji B-cell line. Lanes 11 and 12 are P815 cell lysates probed with the 5927.0 preimmune antiserum (lane 11) or the 5927.2 immune antiserum (lane 12). (b) Immunoprecipitation of U937IF-cell lysates (day 4 of IFN differentiation) with the 5927.2 antiserum (lane 1), preimmune antiserum, 5927.0 (lane 3) or polyclonal antisera generated against other proteins, Hck (lane 4), Lyn (lane 5), Fgr (lane 6), Fps (lane 7) or Csk (lane 8). This blot was probed with the 5927.2 antiserum. In lane 2, the 16 kDa prestained molecular-mass marker is shown. (c) Western-blot analysis of U937IF cells (day 4 of IFN differentiation). Whole-cell lysates were prepared under reducing (lane 1) or non-reducing (lane 3) conditions. The blot was probed with the anti- γ -subunit serum, 5927.2. Lane 2 shows the 16 kDa prestained molecular-mass marker. (d) Anti- γ -subunit immunoblot of anti- γ -subunit immunoprecipitates of U937IF cells (day 4 of IFN differentiation). Lane 1, U937IF-cell lysate immunoprecipitated with preimmune antiserum (5927.0); lane 2, lysate immunoprecipitated with immune antiserum (5927.2); lane 3, lysate immunoprecipitated with 5927.2 antiserum. In lane 3 the 5927.2 antiserum was preincubated with the C-terminal peptide (Figure 1) before being used to probe the Western blot.

Phosphoamino acid analysis

The phosphorylated γ -subunit detected by both autoradiography and γ -subunit Western blotting was subjected to phosphoamino acid analysis. The immunoreactive bands were cut from nitro-

Figure 3 Expression of the γ -subunit correlates with $Fc\gamma R$ signal relay

(a) The respiratory-burst activity in response to PMA and IC stimulation was measured in U937 cells at different stages of IFN differentiation. U937 cells were incubated in the presence of IFN (750 units/ml) for 0, 3 or 4 days. Cells were then harvested and 2×10^6 cells were stimulated with PMA ($1 \mu\text{g/ml}$) or insoluble IC ($50 \mu\text{g}$). The data are expressed as nmol of O_2^- produced as measured by the reduction of cytochrome *c* over a 20 min incubation with or without agonist: day 0: ■, no stimulation; ▨, +PMA; ▩, +IC; day 3: ▤, no stimulation; □, +PMA; ■, +IC; day 4: ▥, no stimulation; ▦, +PMA; ▧, +IC. The bars represent the S.D. of the mean. (b) Anti- γ -subunit immunoblot performed on whole-cell lysates of U937 and U937IF cells. Lysates were prepared from cells differentiated for different times in IFN (750 units/ml) (as described in the Materials and methods section). Proteins were resolved by SDS/PAGE (20% gels) under reducing conditions, transferred to nitrocellulose and probed with the 5927.2 antiserum. Lane M, the position of the 16 kDa prestained molecular-mass marker. Lanes 1–7 show the levels of γ -subunit protein expressed after 0, 1, 2, 3, 4, 5 and 7 days of incubation in IFN respectively. γ^1 and γ^0 denote subspecies of the γ -subunit.

cellulose membranes as described previously [23]. Protein was eluted from the nitrocellulose by incubating the membrane in 50% pyridine/0.1 M ammonium acetate (pH 8.9) (v/v) at 37 °C overnight on a rotator apparatus. The nitrocellulose membrane was removed and the tube was centrifuged at 10000 *g* at room temperature to remove debris. The pyridine/ammonium acetate was removed using a speed vac system, and the eluted protein was hydrolysed in 5.7 M HCl for 1 h at 110 °C. The resulting phosphoamino acids were resolved electrophoretically in two dimensions, exactly as described previously [24]. Carrier phosphoserine, phosphothreonine and phosphotyrosine were added to each sample and detected by spraying the thin-layer plates with a ninhydrin solution. The ^{32}P -labelled phosphoamino acids were visualized by autoradiography using a Phosphor-imaging system (Molecular Dynamics Inc.). The data in Figure 4(c) and Figure 6(b) represent phosphor images of the ^{32}P signal.

RESULTS

Characterization of anti-peptide sera against the γ -subunit

Two rabbits were immunized with the same immunogen and

both antisera (5927.2 and 5932.2) immunoprecipitated a broad 7 kDa protein band (designated γ^0) (Figure 2a, lanes 2 and 4) from U937IF cells. Both antisera also immunoprecipitated a molecule of lower electrophoretic mobility (designated γ^1) on SDS/PAGE in reducing conditions (Figure 2a, lanes 2 and 4). Preimmune antiserum did not immunoprecipitate the γ^1 or γ^0 proteins (Figure 2a, lanes 1 and 3). We have shown in other experiments that preimmune antisera do not detect the γ^1 or γ^0 -proteins in Western-blot analysis of U937IF-cell lysates (results not shown). We compared the amount of γ -subunit expressed in U937 cells (Figure 2a, lane 5) with the amount expressed in U937IF cells (Figure 2a, lanes 6, 7 and 8). We also tested the 5927.2 antiserum on lysates prepared from a Jurkat T-cell line (known to express the ζ -subunit) and a surface immunoglobulin-positive Raji B-lymphoblastoid cell line (Figure 2a, lanes 9 and 10 respectively). These lysates were analysed in parallel and showed no immunoreactivity with the 5927.2 antiserum. The 5927.2 antiserum also recognized the same 7 kDa protein in whole-cell lysates of the P815 mastocytoma cell line, previously known to express the γ -subunit [7] (Figure 2a, lane 12). In this cell line, we did not observe the γ^1 -species. The γ^0 -subunit was

not seen when we probed the same P815 lysate with the preimmune antiserum, 5927.0 (Figure 2a, lane 11).

The preimmune antiserum and several polyclonal rabbit antisera raised against other proteins did not immunoprecipitate this 7 kDa protein. Rabbit antisera against Hck, Lyn, Fgr, Fps and Csk were used to immunoprecipitate these proteins from lysates of U937IF cells. These immunoprecipitates were subjected to SDS/PAGE and probed with 5927.2 antiserum (Figure 2b, lanes 4–8). As a control the same U937IF-cell lysate was immunoprecipitated with the 5927.2 antibody (Figure 2b, lane 1) or with the 5927.0 antibody (lane 3).

On non-reducing SDS/PAGE, the 5927.2 antiserum recognized a doublet migrating at 14 and 15 kDa (Figure 2c, lane 3). These proteins migrated with half the electrophoretic mobility of γ^0 under reducing conditions (Figure 2c, lane 1) and probably represent dimeric γ^0 .

Lastly, a comparison of the immune and preimmune antisera showed that the 5927.2 antiserum immunoprecipitated the γ^1 and γ^0 proteins from lysates of U937IF cells and that the detection of both proteins is totally blocked by preincubation of the Western-blotting antibody with the C-terminal peptide (Figure 2d, compare lanes 2 and 3).

Expression of the γ -subunit correlates with Fc γ R signal relay

The γ -subunit was expressed at very low levels in undifferentiated U937 cells and underwent a marked induction of expression with IFN treatment (Figure 3b, compare lane 1 with lanes 2–7). The predominant form of γ -subunit expressed on days 1–3 of IFN differentiation was γ^0 (Figure 3b, lanes 2–4). On day 4 we observed the emergence of a γ -subunit subspecies which migrated more slowly on SDS/PAGE, referred to as γ^1 (Figure 2a, lanes 2, 4 and 8 and Figure 3b, lanes 5–7). This form of the γ -subunit was up-regulated on day 4 of IFN differentiation and persisted as long as the cells were maintained in IFN. We have observed a constant level of γ^1 -subunit expression from day 4 to day 14 of IFN differentiation (results not shown). The presence of γ^1 -subunit was correlated with the potential to transfer signal via Fc γ R, as measured by quantifying the respiratory burst after IC activation (compare the O_2^{--} -production response to IC stimulation after 3 days of IFN treatment with that on day 4) (Figure 3a). On day 3 of IFN treatment, γ -subunit protein expression had been induced 7-fold, but no signal relay via Fc γ R could be detected (Figures 3a and 3b, lane 4). On day 4, the expression of the γ -subunit was 10-fold above that in undifferentiated U937 cells and the emergence of the γ^1 band was observed (Figure 3b, lane 5). At this stage of differentiation, potent signal transduction via Fc γ R was observed leading to a respiratory burst (Figure 3a). On day 3 we observed no γ^1 -subunit expression and no signalling via Fc γ R (Figure 3a). However, on day 3 of IFN treatment we observed that PMA stimulation activates the respiratory burst of U937IF cells, suggesting that the biochemical components required for respiratory-burst activity are present by day 3 of IFN differentiation (Figure 3a).

Changes in the phosphorylation of the γ^1 -subunit after Fc γ R activation

The γ^1 -band which appeared on day 4 of IFN differentiation underwent a mobility shift in U937IF cells under conditions of Fc γ R activation (Figures 4a and 5b) (compare lane 1 with 2 and 3). This shift in mobility occurred rapidly in IC-stimulated cells and was complete after 1 min of stimulation at 37 °C (Figure 5d, lane 4). The γ^1 -protein appeared as a single band in resting

Figure 4 Phosphorylation of the γ -subunit after Fc γ R activation

(a) the γ -subunit was immunoprecipitated from resting U937IF or U937IF cells stimulated with IC (50 μ g) (day 4 of IFN differentiation). Proteins were resolved by SDS/PAGE (20% gels) under reducing conditions, transferred to nitrocellulose and probed with the 5927.2 antiserum. U937IF cells (differentiated for 4 days in IFN) were metabolically labelled with [32 P] P_i (1 mCi/ml for 4 h) and stimulated with insoluble IC as described in the Materials and methods section. The γ -subunit was immunoprecipitated from resting cells (lane 1) or from cells after stimulation with IC for 1 min (lane 2) or 5 min (lane 3). Immunoreactive species, γ^0 , γ^1 and γ^2 are indicated by arrows. Lane M, the 16 kDa prestained molecular-mass marker. (b) Autoradiogram of the Western blot shown in (a) (exposure 6 h). The phosphorylated γ^1 -protein is observed to split into γ^{1a} and γ^{1b} subspecies as labelled. (c) Phosphoamino acid analysis of the phosphorylated γ^1 -subunit. The γ^1 bands were cut from the nitrocellulose membrane shown in (a) and (b), eluted and subjected to phosphoamino acid analysis. (1) γ^1 immunoprecipitated from resting U937IF cells; (2) and (3) γ^1 immunoprecipitated from U937IF cells after IC stimulation for 1 min and 5 min respectively. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

U937IF cells and was converted into a doublet after Fc γ R activation, designated γ^{1a} and γ^{1b} (Figure 4a, compare lanes 1 and 2, Figure 5d, compare lanes 1 and 4, Figure 6a, lane 2). To determine if this mobility shift in the γ^1 -subunit was associated with changes in phosphorylation of this subunit, U937IF cells were metabolically labelled using [32 P] P_i followed by activation of their Fc γ R. These experiments demonstrate that the γ^1 protein is phosphorylated in the resting U937IF cell and undergoes a qualitative and quantitative change in phosphorylation after Fc γ R activation (Figure 4b, lanes 1–3). The γ^1 -protein splits into γ^{1a} and γ^{1b} subspecies, as indicated by the formation of a doublet (Figure 6a, lane 2). Phosphoamino acid analysis of the γ^1 -subunit before and after Fc γ R activation demonstrated that the γ^1 -protein is threonine phosphorylated in the resting cell and that the shift in mobility observed in the γ^1 -protein was associated predominantly with serine phosphorylation of this molecule (Figure 4c). Therefore the mobility shift observed on Western-blot analysis of the γ^1 -subunit predominantly reflects an increase in serine phosphorylation. In addition, at a later stage of Fc γ R activation we also observed tyrosine phosphorylation of γ^1 -subunit (Figure 4c, panel 3). If we excised the individual γ^{1a} and

Figure 5 Effects of Fc γ R and PMA stimulation on the mobility of the γ -subunit and respiratory burst in U937 and U937IF cells

(a) Respiratory-burst (O_2^{--} production) response is measured in U937 cells (2×10^6 cells) after PMA ($\bullet$; $1 \mu\text{g/ml}$) or IC ($\blacksquare$) stimulation; $\blacktriangle$, no stimulation. The data are expressed as nmol of O_2^{--} produced over time. The bars represent S.D. of the mean. (b) Anti- γ -subunit immunoblot of anti- γ -subunit immunoprecipitates of U937 cells. The γ -subunit was immunoprecipitated from resting U937 cells (lane 1) or U937 cells stimulated with PMA ($1 \mu\text{g/ml}$) for 1 (lane 2) or 5 (lane 3) min. Lane 4, cells were stimulated with IC ($50 \mu\text{g}$) for 1 min. Lane 5, immunoprecipitation of the γ -subunit from a resting population of U937IF cells (day 4 of IFN differentiation). Immunoprecipitated proteins were resolved by SDS/PAGE (20% gels), transferred to nitrocellulose and probed with the 5927.2 antiserum. γ^0 and γ^1 subspecies are shown with arrows. Lane M, the 16 kDa prestained molecular-mass marker. (c) Respiratory-burst (O_2^{--} production) response is measured in U937IF cells (2×10^6 cells) after PMA ($\bullet$; $1 \mu\text{g/ml}$) or IC ($\blacksquare$) stimulation; $\blacktriangle$, no stimulation. The data are expressed as nmol of O_2^{--} produced over time. The bars represent S.D. of the mean. (d) Anti- γ -subunit immunoblot of anti- γ -subunit immunoprecipitates of U937IF cells (day 4 of IFN differentiation). The γ -subunit was immunoprecipitated from resting U937IF cells (lane 1) or from U937IF cells stimulated with PMA for 1 (lane 2) or 5 (lane 3) min. Lanes 4 and 5 represent U937IF cells stimulated with IC for 1 and 5 min respectively. γ^0 and γ^1 immunoreactive species are shown with arrows, and the γ^{1a} and γ^{1b} subspecies are labelled. Lane M, the 16 kDa prestained molecular-mass marker.

γ^{1b} subspecies after 1 min of FcR stimulation and performed phosphoamino acid analysis on these proteins, we observed that the electrophoretically more mobile γ^{1b} -protein is predominantly phosphorylated on serine (Figure 6b) and the γ^{1a} -protein is predominantly phosphorylated on threonine (Figure 6a, lane 2, Figure 6b). The kinetics of phosphorylation of γ^1 -protein in response to FcR activation is slightly different in Figures 4(a), 4(b) and Figure 5(d). This may be explained by the different conditions of IC stimulation used during the metabolic labelling

experiments, in that the metabolically labelled cells were stimulated at room temperature for 1 or 5 min for radiation safety considerations. The U937IF cells in Figure 5(d) (lanes 4 and 5), were stimulated at 37°C . At this temperature, the conversion of γ^1 into γ^{1a} and γ^{1b} occurs more rapidly going to completion in 1 min. At 37°C the further incubation of U937IF cells in the presence of IC results in conversion of γ^{1b} into γ^{1a} (results not shown). We have also observed one additional phosphoprotein in anti- γ -subunit immunoprecipitates migrating above the γ^1 -

Figure 6 Phosphorylation of γ^{1a} and γ^{1b} proteins

(a) U937IF cells (differentiated for 4 days in IFN) were metabolically labelled in [32 P] P_i (1 mCi/ml for 4 h) and stimulated with insoluble IC or not stimulated. The γ -subunit was immunoprecipitated from resting untreated cells (lane 1) or from cells stimulated with IC for 5 min (lane 2). (b) The individual γ^{1a} and γ^{1b} bands were cut from the nitrocellulose membrane, eluted and subjected to phosphoamino acid analysis as described in the Materials and methods section. (1) Analysis of the γ^{1a} band cut from lane 2 of (a); (2) analysis of the γ^{1b} band cut from lane 2 of (a). S, Phosphoserine; T, phosphothreonine.

band in FcR-stimulated cells (designated γ^2). This protein shows anti- γ -subunit immunoreactivity (Figures 4a and 4b, lanes 2 and 3). The presence of this γ^2 -subunit immunoreactive species is not as reproducible a finding as the presence of the γ^0 and γ^1 molecules.

In contrast with γ^1 -protein, the majority of the γ -subunit (γ^0) was not phosphorylated in U937IF cells and was not phosphorylated in response to Fc γ R activation (Figure 4b, lanes 1–3). This is further supported by the lack of observation of a γ^1 -band in U937IF cells differentiated with IFN for 3 days and stimulated with IC (results not shown). Metabolic labelling experiments have not demonstrated [32 P] P_i labelling of the major γ^0 protein band (Figure 4b, lanes 1–3). An analysis by densitometry based on the amount of γ^1 and γ^0 protein present in U937IF cells on day 4 of IFN differentiation shows that 5% of total γ -subunit protein is phosphorylated in these myeloid cells.

Phosphorylation of the γ -subunit is activated by protein kinase C (PKC)

The γ -subunit is predominantly serine-phosphorylated in response to Fc γ R activation. To determine if PKC is involved in this serine-phosphorylation event, we stimulated U937 and

U937IF cells with PMA or IC and immunoprecipitated the γ -subunit from these cells and subjected it to anti- γ -subunit Western-blot analysis. In parallel experiments, the relative responsiveness of the U937 and U937IF cells to PMA and IC stimulation was determined (Figures 5a and 5c). An identical mobility shift occurred in the γ^1 -protein after PMA or Fc γ R stimulation (Figure 5d, lanes 1–5). No mobility shift was observed in the γ^0 subunit of undifferentiated U937 cells after PMA or Fc γ R activation (Figure 5b, lanes 1–5). U937IF cells differentiated for 3 days in IFN and stimulated with IC also showed no mobility shift in γ^0 -subunit and no appearance of γ^1 -subunit (D. L. Durden, H. Rosen and J. A. Cooper, unpublished work). In U937IF cells, the kinetics of the mobility shift differed for PMA and Fc γ R stimulation. PMA stimulation resulted in a slower and more prolonged shift in the γ^1 -protein occurring over a 5 min incubation (Figure 5d, lanes 1–3). In other experiments, we have noted that the mobility shift from γ^{1a} to γ^{1b} continues to occur after 10 min of stimulation with PMA. In contrast, the γ^{1a} to γ^{1b} shift occurring after Fc γ R activation was rapid and complete by 1 min of incubation at 37 °C (Figure 5d, lanes 4 and 5). One interpretation of these data is that FcR cross-linking induced by IC more efficiently co-localizes members of the signalling complex (Fc γ R, PKC and γ^1 -subunit), thereby increasing the effective concentration of γ -subunit and PKC. This co-localization may promote the rate of catalysis of the γ -subunit by PKC under conditions of FcR cross-linking. Alternatively, activation of the FcR may lead to activation of protein serine/threonine phosphatases in the cell. It is not clear whether the threonine-phosphorylated species of the γ^1 -subunit is a better substrate for serine/threonine phosphorylation. However, the data show that the γ^1 -subunit is threonine-phosphorylated when U937IF cells begin to relay signals via the FcR. In addition, our preliminary experiments suggest that, if U937 cells are stimulated with PMA after 3 days of IFN differentiation, a γ^1 -subunit emerges. In these same cells, FcR stimulation is not associated with the appearance of a γ^1 -subunit and these cells do not signal via the Fc γ R. These data suggest that the unphosphorylated γ^0 form of the γ -subunit can be a substrate for PKC. Alternatively, the effects of PMA stimulation on the phosphorylation of the γ^0 - or γ^1 -protein could be indirect involving activation of other serine/threonine kinase(s) (e.g. mitogen-activated protein kinase, raf, etc.).

MAb cross-linking of Fc γ RI induces the phosphorylation of γ -subunit

MAbs specific for different classes of Fc γ R were used to cross-link these receptors on U937IF cells. The shift in mobility of the γ -subunit only occurred in cells activated through Fc γ RI. Three different mAbs specific for Fc γ RI, namely mAbs 32, 22 and 197 [7], were used. All three induced the appearance of the same γ^1 -band, as determined by anti- γ -subunit immunoblot analysis. MAb 197 induced the largest mobility shift in the γ -subunit (Figure 7a, lane 10). The secondary antibody (rabbit anti-mouse Fab $_2$) alone and mAbs specific for other Fc γ R [mAb 3G8, (Fc γ RIII) and IV.3, (Fc γ RII)], did not induce this mobility shift in the γ -subunit (Figure 7a, lanes 2–9). Stimulation of the same U937IF cells with BSA/anti-BSA IC induced the same mobility shift in γ -subunit (Figure 7b, lane 2). In addition, the specificity of this coupling of γ -subunit phosphorylation to Fc γ RI signalling was supported by the observation that stimulation of U937IF cells with agonists other than PMA (e.g. formylmethionyl-leucyl-phenylalanine and the calcium ionophore, A23187) did not result in a shift in mobility of the γ -subunit (D. L. Durden, H. Rosen and J. A. Cooper, unpublished work).

Figure 7 Effects of FcγRI, FcγRII and FcγRIII cross-linking on the mobility of the γ-subunit

(a) U937IF cells (day 4 of IFN differentiation) were preincubated with primary mAbs specific for FcγRI [mAb 197 (αCD64)]; FcγRII [mAb IV.3 (αCDw32)] or FcγRIII [mAb 3G8 (αCD16)] followed by cross-linking with a rabbit anti-mouse (RαM) secondary antibody as described in the Materials and methods section. All reagents were Fab₂ fragments of IgG except the FcγRII (IV.3) antibody. The γ-subunit was immunoprecipitated from resting (lane 1) or stimulated (lanes 2–11) U937IF cells. Proteins were resolved by SDS/PAGE (20% gels) under reducing conditions, transferred to nitrocellulose and probed with the 5927.2 antiserum. (b) U937IF cells (day 4 of IFN differentiation) were stimulated with insoluble IC (50 μg) (lane 2) or not stimulated (lane 1). The γ-subunit was immunoprecipitated and the proteins were resolved by SDS/PAGE. An anti-γ-subunit immunoblot was performed as described in the Materials and methods section.

DISCUSSION

Protein phosphorylation is important in signal transduction through the FcRs for immunoglobulin [25]. In mast cells, cross-linking of FcεRI stimulates tyrosine phosphorylation of cell proteins, including the γ-subunit [2,11,26]. Eiseman and Bolen [26] have reported that FcεRI is associated with two src-family protein tyrosine kinases, Lyn and Yes, and, on FcεR activation, these enzymes are activated, as demonstrated by an increase in their kinase activity. Huang et al. [27] have reported that cross-linking of FcγRII on platelets and a human erythroleukaemia cell line results in tyrosine phosphorylation of multiple proteins including FcγRII itself. Others [28,29] have reported that cross-linking of FcγRI or FcγRII in U937 cells results in the rapid hydrolysis of PtdIns(4,5)P₂ resulting from tyrosine phosphorylation and activation of phospholipase C-γ1.

FcεRI and the FcγRIIIA (CD16) are non-covalently associated with the γ-subunit, which is presumably involved in the transmission of intracellular signals [12,13]. The observation that the γ-subunit is associated with FcγRIIA in murine macrophages [8] suggested to us that other FcγRs may also relay signals via the γ-subunit. Two laboratories have now reported that FcγRI is non-covalently associated with the γ-subunit of FcεRI [9,10]. We have confirmed these results using our anti-γ-subunit serum (D. L. Durden, H. Rosen and J. A. Cooper, unpublished work).

We have used the U937 cell line to study signal relay through the FcγR. Our line of U937 cells expresses three structurally related but distinct FcγRs, RγRI, FcγRII and FcγRIII [15,30]. Differentiation of U937 cells with IFN induces only a small increase in the expression of FcγRII and FcγRIII (5% increase

in fluorescence intensity) [30]. In contrast, FcγRI is more significantly up-regulated by IFN treatment of U937 cells (25% increase in fluorescence intensity), and this correlates with the capacity to elicit a respiratory burst in response to FcγRI cross-linking [30]. FcεRI is not expressed in U937 cells. We have utilized insoluble IC and mAbs specific to FcγRI, FcγRII and FcγRIII to activate FcγRs in U937 cells.

In this report we show that IFN induces the expression of the γ-subunit of FcγR. IFN is known to prime and activate monocytes and up-regulate FcγRI expression. We are unaware of any reports showing an effect of IFN on any other member of the ARH1 family. We do not know whether the γ-subunit is produced by an IFN-responsive gene as is FcγRI [31]. Expression of the γ-subunit correlates with the capacity to relay signals through FcγR, and IFN induces the expression of a phosphorylated form of the γ-subunit (γ¹). Our results show that the γ¹-protein undergoes a mobility shift under conditions of FcγRI cross-linking, a shift that represents serine phosphorylation. The mAb-cross-linking data provide support for the conclusion that FcγRI relays signals via the γ-subunit of FcεRI. We show that FcγR activation and PMA stimulation of U937IF cells results in serine phosphorylation of the γ-subunit, and that this receptor subunit is a substrate for a serine/threonine kinase in the cell. In other experiments, we have demonstrated that the protein tyrosine phosphatase inhibitor, phenylarsine oxide (10 μg/ml), inhibits the γ^{1a} to γ^{1b} mobility shift and FcR signal transduction, suggesting that this shift in FcγR-stimulated cells requires this enzyme activity (D. R. Durden, H. Rosen and J. A. Cooper, unpublished work). In these experiments, phenylarsine oxide did not inhibit the shift in mobility induced by PMA. We know little

about the function of the γ^1 -protein. It is also possible that the γ^0 - and γ^1 -protein could be novel proteins, perhaps products of alternative splicing, but we have not tested this hypothesis. Interestingly, we can only immunoprecipitate the γ^1 -subunit under SDS detergent conditions (1 % SDS and not 1 % Nonidet P40 or 1 % Triton X-100), whereas γ^0 is easily immunoprecipitated using non-ionic detergents (results not shown). These data suggest that the γ^1 -subunit may be more tightly associated with the cytoskeleton than is the γ^0 -protein.

The exact role of phosphorylation of the γ - and ζ -subunits as it relates to TCR and FcR function remains unclear. One current model proposed to explain how tyrosine phosphorylation of the γ -subunit may regulate signal transduction via Fc γ R suggests that this phosphorylation promotes association of the γ -subunit with cytoplasmic effector molecules (e.g. phosphoinositide 3-kinase, phospholipase C $\gamma 1$, etc), perhaps through interactions involving SH2 domains [12]. The activation of phospholipase C $\gamma 1$ could result in the production of diacylglycerol and the activation of PKC, which may phosphorylate the γ -subunit on serine residues. The high stoichiometry of serine phosphorylation observed may alter the signalling properties of the γ -subunit within the cell. Support for this model includes several recent observations. Clark et al. [4] prepared a glutathione S-transferase fusion protein of two sequences in the mb1 and B29 proteins containing the respective ARH1 domains. The mb1 protein was observed to associate with Fyn, Lyn and phosphoinositide 3-kinase *in vitro*, but the B29 ARH1 domain was found to associate with phosphoinositide 3-kinase only and not Lyn or Fyn. It is unclear whether such an association occurs *in vivo* or whether this association is dependent on phosphorylation. These observations support the contention that these ARH1 domains may transmit signals by association with known and perhaps unknown signal-relay effector molecules, and that some level of specificity may be mediated through the amino acid sequence of the cytoplasmic portions of the ARH1 domains.

The role of threonine and serine phosphorylation in the function of the ARH1 family of proteins is unknown. There are no previous reports of serine phosphorylation of any other member of the ARH1 family; previous reports have mostly described tyrosine phosphorylation. In many systems, serine phosphorylation plays a prominent role in regulation of protein function. Serine phosphorylation of Ick as a consequence of the activation of PKC [32] is thought to interrupt CD4 signal relay by causing the dissociation of Ick from CD4. In our system, the γ -subunit is predominantly serine phosphorylated after Fc γ R activation. This occurs rapidly after FcR activation before tyrosine phosphorylation (Figures 4b and 4c). It is possible that the serine phosphorylation of the γ -subunit may serve to down-regulate γ -subunit receptor function. If so, specific site-directed mutation of the specific serine-phosphorylation sites may serve to generate a more potent signalling molecule. Serine phosphorylation of the γ -subunit has not been observed under conditions of Fc ϵ RI activation [2]. Our data suggest a divergence in the structure/function relationship of the γ -subunit in signal relay as it relates to Fc γ RI, Fc ϵ R and TCR. Future studies in our

laboratory will focus on determining the specific sites of serine phosphorylation within the γ -subunit as a prelude to site-directed mutagenesis of these sites to test which sites contribute to R γ RI signal relay.

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