Interferon-γ Reduces Cell Surface Expression of Annexin 2 and Suppresses the Invasive Capacity of Prostate Cancer Cells*§

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The effect of interferon-γ (IFNγ) treatment on cell surface protein expression was studied in the human prostate cancer cell line 1542CP3TX. IFNγ increased both the number and abundance of proteins in membrane fractions. In contrast, the expression of annexin 2 and its binding partner p11 decreased by 4-fold after 24 h of exposure, with the remaining anx2 complex localized to lipid rafts. Within the same time scale, IFNγ reduced the abundance of the peripherally attached, anx2-associated proteases procathepsin B and plasminogen. The invasive capacity of the cancer cells was reduced by treatment with IFNγ or antibody to annexin 2 in 1542CP3TX cells, but not in LNCaP, an annexin 2-negative prostate cancer cell line. Expression of annexin 2 in LNCaP cells increased their invasiveness. IFNγ induced calpain expression and activation and increased the phosphorylation and degradation of the calpain substrate ABCA1 in 1542CP3TX cancer cells. Surface expression of annexin 2 was reduced in cells treated with glyburide, an ABCA1 inhibitor, whereas inhibition of calpain abrogated IFNγ-induced annexin 2 down-regulation and suppression of Matrigel invasion. The findings suggest annexin 2 externalization is coupled to lipid efflux in prostate epithelium and that IFNγ induces down-regulation of the protease-binding anx2 scaffold at the cell surface and consequently acts to suppress invasiveness through calpain-mediated degradation of the lipid transporter ABCA1.

Prostate cancer is the second most common cause of cancer death in men and the most frequently diagnosed malignancy in men. Whereas the cancer is contained within the prostate, the disease is curable with surgery or radiotherapy, but once it has spread there are no curative treatments. Thus a major challenge is to identify new methods to delay or prevent the progression of the disease.

Cytokines are among the most potent extracellular regulators of protein expression at the cell surface. In addition to regulating major histocompatibility complex processing and presentation pathways, interferon-γ (IFNγ) up-regulates the surface densities of many molecules (1–8) and down-regulates the expression of other surface proteins, including CCL20 receptor CCR6 (9), macrophage CD9 (8), transferrin receptor (10), and interleukin-4 receptor (11).

In vitro studies of prostate cancer cell lines have demonstrated that interferons can reduce the growth rate (12), HER-2 expression (13), basic fibroblast growth factor expression (14), and up-regulate p21 WAF1 (15, 16). IFNγ reduced tumor uptake, growth, and metastasis in an experimental mouse model of prostate cancer (17), and systemic administration of interferons, in combination with other agents in phase I and II trials, has produced biochemical responses (lowering of serum levels of prostate-specific antigen) in patients with hormone-relapsed prostate cancer (18–21).

Annexin 2 is a member of a family of peripheral membrane-binding proteins characterized by their ability to bind to acidic phospholipids in a calcium-dependent manner. This property is shared with two other protein families, namely the pentraxins and vitamin-K-dependent proteins (22). Annexin 2 is unique within the annexin family, as it exists both as a monomer and in a heterotetrameric complex within cells (22). The tetrameric complex is formed by two copies of the 36-kDa annexin 2 molecule bound to a dimer of the p11 protein, a member of the S-100 family of calcium-binding proteins, also referred to as the annexin 2 light chain (23).

Annexin 2 is found on the surface of many cell types, including neurons, leukocytes, monocytes, macrophages, and endothelial cells (24–29). Surface-bound annexin 2 interacts with extracellular matrix proteins such as collagen 1 (30) and tenascin-C (24), and mediates high affinity binding of β2-glycoprotein I to endothelial cells (28). The tetrameric annexin 2-p11 complex also functions as a receptor for tissue-type plasminogen activator and plasminogen, and their simultaneous binding to annexin 2/p11 at the endothelial cell surface results in a 60-fold increase in the catalytic efficiency of plasmin generation (26, 31–33). Increased production of the fibrinolytic serine pro-

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© The abbreviations used are: IFNγ, interferon-γ; OGP, octyl-β-D-glucopyranoside; PBS, phosphate-buffered saline; GFP, green fluorescent protein; LMW, low molecular weight.
Regulation of Cell Surface-exposed Annexin 2

tease plasmin by annexin 2-overexpressing leukocytes is asso-
ciated with hemorrhagic complications in patients with acute
promyelocytic leukemia (29, 34). Annexin 2-mediated plasmin
generation further facilitates matrix degradation and invasion
by macrophages (25) and neurite development in differentiat-
ing PC-12 cells (27).

Up-regulation of annexin 2 on the surface of tumor cells has
been reported in colorectal cancer, breast cancer, pancreatic
cancer, gastric cancer, malignant melanoma, and glioblastoma
multiforme (35–37) and can be associated with poor prognosis
(35, 37). Activated leukocyte cell adhesion molecule and annexin 2
are implicated in the metastatic progression of tumor
cells after chemotherapy with adriamycin (38), and autoanti-
bodies to annexin 2 are frequently observed in patients with
lung cancer (39). Annexin 2 serves as a binding platform for
procathepsin B on the surface of tumor cells (37), and expres-
sion of annexin 2 facilitates tissue plasminogen activator-de-
pendent, plasmin-mediated invasion in breast and pancreatic
cancers (40, 41).

We have used a proteomics based strategy to identify new
cell surface markers in prostate cancer and have identified sev-
eral deregulated membrane proteins by comparing their
expression and regulation by cytokines in normal and cancer
cells (42, 43). Here, long term treatment with IFNγ is shown to
reduce the surface expression of annexin 2 and its binding part-
er p11 in 1542CP3TX prostate cancer cells. In consequence,
the abundance of surface associated pre-proteases such as pro-
cathepsin B and plasminogen is reduced, as is invasiveness.
This study demonstrates a novel anticancer action of IFNγ.

MATERIALS AND METHODS

Cells—The prostate cancer cell line 1542CP3TX was grown
in keratinocyte serum-free medium supplemented with 5%
fetal calf serum, as described by the originators (44). LNCaP
cells were grown in RPMI 1640 medium containing 10% fetal
calf serum.

Surface Labeling, Enrichment, and Separation of Membrane
Proteins—Vectorial labeling with sulfonated succimidyl ester
derivatives of biotin (42) was performed prior to interferon
exposure or at the end of each treatment period. For avidin
affinity chromatography, sulfo-NHS-SS-biotin-labeled cells
were lysed in 1% octyl-β-D-glucopyranoside (OGP) in PBS,
containing a protease inhibitor mixture with or without EDTA
(Complete, Roche Applied Science). Where appropriate, OGP
extractions were performed in the presence of the following
phosphatase inhibitors: 1 μM okadaic acid, 5 μM α-cyano-3-
phenoxybenzyl α-(4-chlorophenyl) isovalerate, and 5 μM
potassium bisperoxo(1,10-phenanthroline) oxovanadate. Pro-
tein concentrations were determined by a modified Lowry
assay (RC DC protein assay, Bio-Rad).

The OGP-solubilized SS-biotin-labeled membrane fractions
were purified by avidin affinity chromatography as described
previously (42). The eluted fractions were dialyzed against dis-
tilled water, vacuum-concentrated, rehydrated in electro-
phoresis buffer, the isolated membrane proteins separated by
SDS-PAGE or two-dimensional gel electrophoresis, and visual-
ized by Coomassie or silver staining. In-gel digestion and mass
spectrometry analysis of the generated peptide mixtures were
performed as described previously (45). In some experiments
the enriched surface fractions were transferred to NC mem-
branes following electrophoresis and probed with horseradish
peroxidase-avidin or antibodies. Avidin- and antibody binding
was detected by the enhanced chemiluminescence technique.
Secondary antibody-alone controls were included and were
negative in all cases.

High salt or chelating dissociation buffers were used to
release peripherally attached proteins from the surface of intact
cells. The cells were washed twice in PBS followed by a phos-
phate buffer with protease inhibitors and either CaCl2 (0.5–15
mM) or EDTA (0.5–5 mM), and surface proteins were released
by gentle swirling. Calcium- and EDTA-free buffer treatments
served as control. After 2 min the dissociation buffer was
removed by aspiration, and the released proteins were concen-
trated by spin-vac centrifugation before being separated by
SDS-PAGE, transferred to blotting membranes, and stained
with antibody. Alternatively, peripherally attached proteins
were released from biotinylated cells by treatment with a hyper-
tonic phosphate buffer containing 0.65 M NaCl and protease
inhibitors, for 5 min at room temperature. The high salt buffer
was removed by aspiration and diluted to isoosmolarity, and
avidin-conjugated agarose beads (20 μl/ml) were added. After
15 min of mixing by rotation, proteins bound to the avidin
beads were isolated by centrifugation. Following five washes in
isotonic PBS, pH 7.4, plus protease inhibitors, the purified pro-
teins were eluted from the beads by heating in a reducing Lae-
mli buffer and separated by SDS-PAGE.

Analysis of ABCA-1 Phosphorylation—[33P]Orthophosphate
labeling was employed to investigate the phosphorylation sta-
tus of ABCA1 in IFNγ-treated cells. Cells were grown to 70%
confluence, washed twice in phosphate-free Dulbecco’s modi-
fied Eagle’s medium containing 0.1% fetal bovine serum, incu-
bated for 1 h in the same medium, and labeled with 0.1 μCi/ml
[33P]orthophosphate for 6 h prior to exposure to interferon.
After different stimulation periods, the cells were lysed in RIP-
A buffer containing 1% Nonidet P-40 and 0.2% deoxycholate in
PBS, pH 7.4, plus the protease and phosphatase inhibitor
mixtures. ABCA1 and associated binding partners were then
isolated by immunoprecipitation with monospecific, purified
rabbit antibody (Novus Biologicals NB 400-105). Following
SDS-PAGE separation, the immunopurified radiolabeled pro-
teins were transferred onto polyvinylidene difluoride mem-
branes, dried, and visualized by PhosphorImaging on a Molecu-
lar Imager FX scanner (Bio-Rad).

Live Cell Immunofluorescence Staining—Live cell immuno-
fluorescence was performed using the protocol of Groves (46).
Live cells were initially incubated with primary antibodies
against annexin 2 and p11 and with cholera toxin. The cells
were then fixed, permeabilized, and incubated with phalloidin
to visualize the actin cortex, and finally stained with fluoro-
phore-conjugated secondary antibodies.

Cell Invasion Assays—The Biocoat Matrigel invasion cham-
er system (BD Biosciences) was used to assay the invasive
potential of cells cultured in the presence or absence of IFNγ,
employing the protocol of Falcone et al. (25). To evaluate the
effect of antibody blockage, the Matrigel was re-hydrated in
normal medium or in a medium containing 25 μg/ml mouse
monoclonal antibody against annexin 2 (catalog number 034400, Zymed Laboratories Inc.) at 4 °C. The same concentration of an isotypic mouse monoclonal anti-rabbit IgG antibody (Amersham Biosciences) was used as control. A similar protocol was used to assay the invasive effects of annexin 2-GFP transfaction, with the exceptions that the cells here were obtained from a 24-well plate, and the migrants were counted using a fluorescence microscope. To study the effect instigated by inhibition of calpain, 1542CP3TX cells were pretreated for 3 h with 30 μM type-II calpain inhibitor (Calbiochem) or with a control solution of 0.01% Me2SO. The Biocoat Matrigel wells were rehydrated in media containing either Me2SO, IFNγ + calpain inhibitor + Me2SO, calpain inhibitor + Me2SO, or IFNγ + Me2SO, to ensure that seeded cells remained in the appropriate stimulant conditions during subsequent phases of the assay. After 22 h of incubation, the cells were fixed with 4% paraformaldehyde and stained with Hoechst. Migrants were quantified by fluorescence microscopy.

Transfection of LNCaP Cells with a GFP-Annexin 2 Fusion Construct—LNCaP cells were transfected with a plasmid encoding an annexin 2-GFP fusion protein or with the GFP vector alone as a control, to examine how expression of annexin 2 affected their invasive capacity. A pEGFP-C1 vector-based wild type annexin 2 construct (47) was transfected into LNCaP cells using the FuGENE transfection reagent (Roche Applied Science) twice at 18-h intervals, and cultures demonstrating high GFP and GFP-annexin 2 expression after 48 h were used in the Matrigel invasion assay described above.

RESULTS

IFNγ Reduces Cell Surface Expression of Annexin 2—Vascular labeling with sulfo-N-hydroxysuccinimidyl derivatives of biotin was combined with two-dimensional gel electrophoresis and avidin affinity blotting to study the effect of IFNγ treatment on surface composition in 1542CP3TX prostate cancer cells. IFNγ increased the number of biotinylated proteins and enhanced the membrane density of several surface proteins (Fig. 1A). Relatively few protein spots were down-regulated by IFNγ treatment (upward arrows in Fig. 1A), some because of altered abundance of the individual isoforms in charge trains (white rectangles in Fig. 1A).

To identify proteins regulated by IFNγ and achieve optimal representation of hydrophobic and high molecular weight membrane proteins, avidin affinity-purified surface proteins were separated by SDS-PAGE prior to analysis by mass spectrometry (42). Although IFNγ increased both the repertoire and abundance of proteins in the avidin-isolated biotinylated membrane fractions, the surface density of an abundant 36-kDa protein was strongly reduced after 72 h of exposure to the cytokine (oblique arrows in Fig. 1B). The protein was identified as annexin 2 by matrix-assisted laser desorption/ionization-mass spectrometry analysis of peptides generated by trypsinization of the excised Coomassie-stained gel band (Fig. 1C).

Densitometry analysis of gel images from three independent experiments revealed that the surface expression of the 36-kDa annexin 2 band was reduced by 4-fold after 72 h of IFNγ treatment. Immunoblotting of avidin-purified biotinylated membrane fractions from treated and untreated 1542CP3TX cells confirmed the identity of the 36-kDa band and the reduction in annexin 2 surface expression induced by IFNγ (inset in Fig. 1C). A 54-kDa protein that was strongly up-regulated in both whole cell extracts and membrane fractions from IFNγ-treated cells (horizontal arrow in Fig. 1B) was identified as interferon-induced protein 53 (IFP53) by mass spectrometry (data not shown).

Annexin 2 was readily released from the cell surface by treatment with chelating or high salt dissociation buffers, suggesting that the protein is peripherally bound to the plasma membrane in a calcium-dependent manner (supplemental Fig. 1). To study the kinetics of annexin 2 down-regulation, EDTA-released membrane fractions were obtained at various times after addition of IFNγ and analyzed by immunoblotting. Although the membrane density of annexin 2 remained high during the first 2 h of IFNγ treatment, it was strongly reduced after 4 h (Fig. 1D). Following 6 h of exposure to IFNγ, annexin 2 was barely detectable in EDTA-released surface fractions from 1542CP3TX cells (Fig. 1D and Fig. 4H).

Long term IFNγ treatment did not alter the abundance of annexin 2 in whole cell detergent extracts from 1542CP3TX cells (Fig. 1E). The global expression levels of annexin 2 in the benign prostate epithelial cell line PRE 2.8 and in the prostate cancer cell lines PC3 and DU145 were likewise unaffected by cytokine treatment (supplemental Fig. 2). In accordance with previous findings (48), annexin 2 was not detected in LNCaP prostate cancer cells (supplemental Fig. 2). Taken together, these findings show that IFNγ induces a significant reduction in the membrane density of annexin 2 in 1542CP3TX prostate cancer cells, and that the suppressive effect is specific for peripherally attached surface proteins.

IFNγ Alters the Surface Localization of Annexin 2—The effects of IFNγ were next analyzed by surface staining of intact, viable cells. Cholera toxin was used to stain GM1 gangliosides, which are present in high abundance at lipid rafts, and phalloidin was used to stain F-actin after membrane permeabilization.

Immunofluorescence staining with an antibody against annexin 2 confirmed that the protein is present on the surface of 1542CP3TX cells and that 24 h of treatment with IFNγ strongly reduces the membrane density of annexin 2 (Fig. 2A). In addition to the reduction in peripheral expression, IFNγ altered the distribution of annexin 2 on the cell surface. Whereas a generalized pattern of annexin 2 staining was observed over the surface of unstimulated cells, IFNγ treatment concentrated the remaining annexin 2 molecules to lipid rafts (Fig. 2A).

Whereas the intensity of p11 surface staining also was strongly reduced following 24 h of IFNγ treatment, the localization of p11 remained unaltered closely mirroring that of the cholera toxin-positive lipid raft structures in both treated and untreated cells. 24 h exposure to IFNγ did not reduce the global expression level of p11 (supplemental Fig. 3), indicating that the suppressive effect of the cytokine again is specific for proteins expressed on the cell surface.

One possible interpretation of these observations is that annexin 2 exists both as a monomer and in heterotetrameric complex with p11 on the surface of 1542CP3TX cells, and although the monomer binds to membrane lipids outside raft
areas and is more susceptible to IFNγ-induced down-regulation, the heterotetramer is situated within the lipid rafts.

IFNγ Suppresses the Invasive Capacity of Prostate Cancer Cells—To investigate whether IFNγ treatment affects the invasiveness of prostate cancer cells, we compared 1542CP3TX and LNCaP cells in a Biocoat Matrigel invasion chamber assay. LNCaP cells do not express annexin 2 as a result of promoter silencing by hypermethylation (48) (supplemental Fig. 2). Invasion was significantly reduced (p = 0.029) in 1542CP3TX cells after interferon treatment, but LNCaP cells were unaffected (Fig. 3A). Exposure to an antibody against annexin 2 significantly reduced the number of transmigrating 1542CP3TX cells (Fig. 3B), whereas a similar concentration of mouse IgG, raised against rabbit immunoglobulin, had no effect (Fig. 3C).

To confirm that annexin 2 expression influences the invasiveness of prostate cancer cells, annexin 2 was expressed in LNCaP cells as a GFP fusion protein. Although the annexin 2-GFP fusion protein was most abundant in the nucleus and cytosol, a peripheral staining pattern indicative of membrane-localized proteins was seen in most of the transfected cells (supplemental Fig. 4). A graphic representation of data from six independent experiments is shown in Fig. 3D, demonstrating a significant increase (p < 0.002) in the invasive capacity of LNCaP cells transfected with annexin 2 compared with empty vector alone.

Consistent with the heterotetrameric anx2, complex function as an anchorage and activation site for procathepsin B and plasminogen on cell surfaces (37, 25, 30), IFNγ treatment reduces the density of peripherally attached procathepsin B and plasminogen at a similar rate to annexin 2 in 1542CP3TX cells (Fig. 3E). This finding suggests that IFNγ suppresses the invasive capacity of 1542CP3TX prostate cancer cells by reducing...
the surface expression of the heterotetrameric anx2 complex, its associated preproteases, their activation, and the activity of the proteolytic enzymes they in turn control in the pericellular space.

**Regulation of Annexin 2 Surface Expression**—The mechanism(s) responsible for externalization of annexin 2 remains to be determined. Glucocorticoids promote externalization of annexin 1 in folliculostellate cells (49) and the ATP-binding cassette transporter A1 (ABCA1) has been identified as a candidate likely to be responsible for its translocation (50). IFNγ was recently shown to down-regulate cholesterol efflux and ABCA1 expression in macrophage-derived foam cells, whereas the cytokine had no effect on cholesterol transport or ABCA1 expression in foam cells from Stat1 knock-out mice, indicating IFNγ-induced suppression of ABCA1 is mediated by the Stat1 pathway in foam cells (51).

Based on these findings we next investigated whether annexin 2 is susceptible to glucocorticoid treatment and whether its externalization involves ABC transporter activity in the prostate. Annexin 2 content was measured in EDTA-released cell surface fractions from 1542CP3TX cells grown in the presence of either or both the sulfonylurea glyburide, an ABCA1 inhibitor, and dexamethasone, a highly active and stable glucocorticoid.

**FIGURE 2.** IFNγ reduces the density and alters the distribution of annexin 2 and p11 antigens on the surface of prostate epithelial cells. Immunofluorescence analysis of interferon-induced changes in the surface distribution of annexin 2 (A) and p11 (B) in 1542CP3TX cells. Intact viable cells were stained with antibody against annexin 2 (BD Biosciences), p11 (BD Biosciences), or cholera toxin (Molecular Probes) analyzed by fluorescence microscopy and photographed. The cells were then fixed and permeabilized, and the actin cortex was visualized by phalloidin staining. The surface topography of annexin 2 and p11 antigens and their relationship to cholera toxin-stained raft areas and the actin cytoskeleton are presented in merged images in the bottom right corner. Annexin 2 is shown in green, cholera toxin corresponding to lipid raft regions in blue, and actin in red.

**FIGURE 3.** Surface activity of annexin 2 and its associated hydrolases regulates the invasive capacity in annexin 2-expressing prostate cells. A, CP3TX and LNCaP cells were exposed to 500 units/ml IFNγ for 24 h and used to study cell invasion. The migrating cells from six replicate experiments were counted, showing a statistically significant reduction in 1542CP3TX cell invasiveness with IFNγ. The invasive capacity of the annexin 2 nonexpressing LNCaP cells was unaffected by IFNγ treatment. B, pretreatment of the cells and the invasion chamber matrix with antibody against annexin 2 (anx2) reduces the invasive capacity. The data resulting from six replicate experiments are presented. A statistically significant reduction (p < 0.02) in the invasive capacity of 1542CP3TX cells was achieved with annexin 2-specific monoclonal antibody (mAb) (Zymed Laboratories Inc.). C, addition of similar concentration of an isotypic monoclonal antibody against rabbit IgG (scramble antibody) had no effect on invasiveness. D, re-expression of annexin 2 as a GFP fusion protein significantly increased the invasive capacity of LNCaP cells (data tabulated from six replicate experiments). E, immunostaining of CaCl2 released peripheral membrane proteins demonstrating similar reduction in the abundance of surface-associated annexin 2, pro-cathepsin B, and plasminogen in 1542CP3TX cells after 3 h of IFNγ treatment. IB, immunoblot.
Similar to their effects on the translocation of annexin 1, glyburide (100 μM) decreases and dexamethasone (0.1 μM) enhances surface expression of annexin 2 (Fig. 4A and supplemental Fig. 5). When 1542CP3TX cells were exposed to both agents simultaneously, the surface expression of annexin 2 was similar to that of cells treated with dexamethasone alone (Fig. 4A). However, dexamethasone treatment did not reverse the IFN-γ-induced suppression of annexin 2 expression in 1542CP3TX cells (supplemental Fig. 5). The surface density of monomeric annexin 2 began to decrease after 2 h of glyburide treatment (Fig. 4C), and after 24 h both monomeric and tetrameric annexin 2 had almost disappeared from the surface of the cancer cells (Fig. 4B). Treatment with glyburide did not affect the global expression of annexin 2 in 1542CP3TX cells (Fig. 4C). These results show that the surface density of annexin 2 is susceptible to the inhibition of ABC transporter activity, and that glucocorticoid promotes externalization of annexin 2 in prostate epithelium.

IFN-γ reduces the expression of full-size ABCA1 during the first 4 h, whereas the abundance of low molecular weight (LMW) ABCA1 forms gradually increase over the same treatment period (Fig. 4D). Isolation of ABCA1 from [33P]orthophosphate-labeled 1542CP3TX cells showed that phosphorylation of the lipid transporter increases in a similarly
Regulation of Cell Surface-exposed Annexin 2

To corroborate the hypothesis that IFNγ suppresses annexin 2 externalization by promoting calpain-mediated down-regulation of ABCA1, we monitored the surface abundance of annexin 2 in cells stimulated with IFNγ in the presence or absence of type II calpain inhibitor. Treatment with calpain inhibitor did not affect IFNγ-induced JAK-STAT signaling (Fig. 4G), but it completely negated the suppressive effect of interferon on annexin 2 surface expression in 1542CP3TX cells (Fig. 4H). Inhibition of calpain additionally diminished the suppressive effect IFNγ instigates on Matrigel invasion (Fig. 4I), supporting the notion that surface-associated annexin 2 participates in the regulation of invasiveness in prostate cancer cells.

These results show that IFNγ induces phosphorylation and promotes degradation of ABCA1 in 1542CP3TX prostate cancer cells. The IFNγ-induced reduction of annexin 2 surface expression and the suppression of invasiveness were abolished by treatment with calpain type II inhibitor, confirming the involvement of a cysteine protease in the regulatory pathway.

The absence of low molecular weight annexin 2 antigen fragments in the culture medium from interferon-treated cells excludes the possibility that annexin 2 is removed from the cell surface by proteolysis mediated by external cysteine protease such as cathepsin B. Taken together these findings imply that annexin 2 externalization is coupled to lipid traffic and that IFNγ induces down-regulation of peripheral annexin 2 through calpain-mediated degradation of phosphorylated ABCA1 in 1542CP3TX cells.

DISCUSSION

This study describes a new mode-of-action for interferon-γ, which enables the cytokine to modulate cell surface-associated hydrolase activity by regulating the surface density and localization of annexin 2. Interferon reduces the expression of annexin 2 and p11 on the surface of human prostate epithelial cells, leading to a similar reduction in the abundance of the associated pre-proteases procathespin B and plasminogen and suppression of the invasive capacity of 1542CP3TX prostate cancer cells.

Cathepsin B and plasmin are key participants in both the initiation and coordination of surface-associated proteolytic cascades, which facilitate cell migration and invasion. The two hydrolases are also responsible for ECM remodeling during invasion (e.g. generation of new specific attachment sites to promote traction) and participate in the degradation of restrictive structures such as basement membranes. A reduction in the quantity of theirzymogens at the leading edge of migrating cells and the consequent suppression of the activity of the enzymes they in turn control in the pericellular space (e.g. MMP-9 (53)) would therefore explain the decreasing invasive capacity of 1542CP3TX prostate cancer cells following IFNγ treatment.

IFNγ enhances the expression and proteolytic processing of calpain and induces a gradual increase in phosphorylation and degradation of ABCA1 in 1542CP3TX cells. Expression of

FIGURE 4.

Regulation of annexin 2 surface expression by IFNγ. A, 1542CP3TX cells were grown to 70% confluence and treated with either or both glyburide (100 μM for 24 h) and dexamethasone (0.1 μM for the last 3 h). Cells grown for a similar period in the presence of the solvent(s) (0.01% MeSO4 (DMSO) and/or 0.1% EtOH) served as controls. Following treatment the cells were washed three times in PBS before peripherally attached annexin 2 was released from the surface by 0.5 mMEDTA treatment, separated by SDS-PAGE, and visualized by immunoblot. Actin served as a control for whole cell extract loading. B, immunoblot detection of annexin 2 in EDTA-released membrane fractions from untreated 1542CP3TX cells and cells grown in the presence of 100 μM glyburide for 24 h. C, 1542CP3TX cells were cultured to 70% confluence and exposed to 100 μM glyburide for 0 (MeSO4 only), 20, 40, 60, 120, and 240 min. Following three washes in PBS the EDTA-susceptible membrane fractions were released and concentrated. The chelate-treated cells were then solubilized in 1% OGP. The samples were separated by SDS-PAGE, and the 36-kDa form of annexin 2 determined by immunoblot. The experiment was repeated in triplicate, and the blots are representative for the average abundance of annexin 2 in the 12 samples. D, immunoblot (IB) detection of annexin 2 in EDTA-released membrane fractions from untreated 1542CP3TX cells and cells grown in the presence of 100 μM glyburide for 24 h. E, 1542CP3TX cells were cultured to 70% confluence and exposed to 100 μM glyburide for 0 (MeSO4 only), 20, 40, 60, 120, and 240 min. Following three washes in PBS the EDTA-susceptible membrane fractions were released and concentrated. The chelate-treated cells were then solubilized in 1% OGP. The samples were separated by SDS-PAGE, and the 36-kDa form of annexin 2 (ann2) was detected by immunoblot. 30 μg of whole cell lysate was loaded per well. This experiment was conducted in triplicate, and a representative result is presented. Actin served as a control for whole cell extract loading. F, ABCA1 expression in IFNγ-treated 1542CP3TX cells demonstrated by immunostaining of whole cell detergent extracts. The expression of the 220-kDa full-size ABC transporter A1 is down-regulated by 30–40% after 1 h and further reduced after 4 h of stimulation, whereas the abundance of low molecular weight ABCA1 forms gradually increases over the same period. G, 1542CP3TX cells were grown to 70% confluence and metabolically labeled with [32P]orthophosphate for 6 h prior to stimulation. The isotope-containing culture medium was then removed and exchanged with cold, phosphate-free medium, and the cells were treated with 500 units/ml IFNγ. After different stimulation periods the cells were lysed in RIPA buffer, and ABCA1 was isolated by immunoprecipitation (IP). ABCA1 and co-precipitated proteins were then separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, dried, and visualized by PhosphorImaging on a Molecular Imager FX scanner (Bio-Rad). The two LMW phosphoproteins indicated by arrowheads are of similar size as two of the LMW ABCA1 antigens detected by immunoblot of whole cell extracts (D). The abundance of calpain 2, large catalytic subunit, demonstrated by immunoblot of whole cell OGP extracts with a monospecific, polyclonal antibody to calpain 2 (C-19, Santa Cruz Biotechnology). Note the disappearance of the 80-kDa calpain 2 form and the increased abundance of lower molecular weight calpain forms following interferon treatment. H, Abundance of ABCA1 in whole cell detergent extracts from cultures treated with IFNγ in the presence or absence of 30 μM type II calpain inhibitor (Calbiochem). Immunostaining of β-tubulin served as loading control. I, Immunoblot of EDTA released membrane fractions from 1542CP3TX cells treated with IFNγ in the presence or absence of calpain inhibitor, l, graphic representation of data from Biocoat Matrigel invasion chamber analysis. 1542CP3TX cells were treated with 30 μM type II calpain inhibitor or 0.01% MeSO4 (control solution) prior to exposure to interferon and seeding into invasion chamber wells that had been pretreated in stimulant condition-specific manner. The cells were incubated for 22 h, fixed with 4% paraformaldehyde, and stained with Hoescht. Migrants were quantified using fluorescence microscopy. Each condition was analyzed in triplicate.
annexin 2 in LNCaP cells by transfection significantly increases their invasive capacity, verifying the role of the protein in the regulation of invasiveness in prostate cancer cells. Calpain type II inhibitor negates the IFNγ-induced annexin 2 down-regulation and suppression of Matrigel invasion, whereas glyburide reduces the surface expression of annexin 2. These results imply that changes in annexin 2 expression and topology are secondary to adjustments in cellular metabolism, trafficking, and/or distribution of cholesterol and phospholipids. IFNγ has been shown to elicit a Ca2⁺ flux in certain cell types (54), and μ-calpain activation by the interferon-inducible chemokine Protein 9 was recently shown to occur through a PLCβ-mediated Ca2⁺ flux in keratinocytes (55). This suggests that although the IFNγ-induced suppression of annexin 2 in 1542CP3TX prostate cancer cells may be initiated by increased calcium entry, it is actuated by a reduction in lipid efflux. Delin- eation of the IFNγ-calpain-ABC1-anx2-plasminogen pathway thus establishes a new link between Ca2⁺ signaling, lipid transport, and the activity of surface-associated proteases.

Annexin 2-associated tissue-type plasminogen activator is thought to regulate the activation of p11-bound plasminogen at the heterotetramer. However, inactive procathepsin B is normally processed to active single and double chains form of cathepsin B in late endosomes and lysosomes, respectively (56). The environment of both these compartments is more acidic than that found at the cell surface, and although the binding of secreted procathepsin B to p11 is thought to facilitate its conversion into cathepsin B (56), the mechanism responsible for activation of anx2t-associated procathepsin B has remained elusive.

Annexins are key regulators of endocytic and exocytic membrane traffic, and association of the anx2t complex with recycling endosomes is facilitated by cholesterol-stabilized membrane domains (57). More notably, the heterotetrameric complex was recently shown to control the distribution of transferrin receptor-containing recycling endosomes in HeLa cells (58). ABC1A has been implicated in the mobilization of cholesterol and sphingomyelin from late endosomes (59). A study of late endocytic vesicles in Tangier disease suggests that internalized ABC1A traffics to late endosomes where it converts late endocytic pools of cholesterol that retain Niemann-Pick C1 protein to pools that together with phospholipids can associate with apoA-I. The “lipated” apoA-I in late endocytic vesicles then traffics back to the cell surface and is released as the nascent high density lipoprotein particle (60). Janus kinase 2 has been shown to modulate the interactions between apoA-I and ABC1A that are required for removal of cellular cholesterol (61). Based on these findings and our observation that internalized anx2t, is recycled back to the cell surface, it is tempting to speculate that the heterotetramer may shuttle procathepsin B between the cell surface and late endosomes. Regulation of procathepsin B’s endocytic trafficking by a combination of annexin 2 (62) and ABC1A (63) activities would further ensure that following its activation in late endosomes the protease is recycled back to the cholesterol and phospholipid-rich membrane domains, above which it exerts its activity.

Annexin 2 has been shown previously to act as a co-receptor for human cytomegalovirus, enabling the virion to anchor to the cell surface prior to entry (64, 65). This implies that IFNγ triggers both anti-invasive and anti-viral actions by suppressing the surface density of annexin 2 in epithelial cells.

IFNγ action is regulated in two distinct ways, control of IFNγ production and modulation of IFNγ signaling. Several mechanisms negatively regulate IFNγ signaling, including down-regulation of IFNγR expression and induction of suppressors of cytokine signaling, which are activated by interferon itself. IFNγ activates macrophages, and it has been proposed that the sensitivity of macrophages to IFNγ is regulated by the opposition of STAT1 and suppressors of cytokine signaling proteins that are expressed in different proportions, depending upon the intensity or duration of an activating stimulus (66). IFNγ also induces the expression of early response genes through activation of the Janus tyrosine kinase/signal transducer and activator of transcription 1 (JAK1/2-Stat1) pathway. IFNγ regulates a variety of other signaling cascades in addition to this pathway. However, little is known about how these signal circuits are activated and contribute to the biological activity of IFNγ (66), and the composition of several remains ill defined.

In conclusion, the functional coupling between intracellular interferon signals and extracellular protease effectors described in this study demonstrates a new mechanism in IFNγ signaling, which may explain how the cytokine is able to modulate the integration of simultaneous incoming extracellular signals during the initial stages of an inflammatory response.

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