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GRANT NUMBER DAMD17-94-J-4349

TITLE: Use of Cytokines to Prevent Breast Cancer Growth and Progression

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REPORT DATE August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (1 Aug 96 - 31 Jul 97)

4. TITLE AND SUBTITLE
Use of Cytokines to Prevent Breast Cancer Growth and Progression

5. FUNDING NUMBERS
DAMD17-94-J-4349

6. AUTHOR(S)
Philip M. Grimley, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation for the
Advancement of Military Medicine
Rockville, Maryland 20852

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Goals of studies with human breast cancer cells have included: (1) characterization of a novel transcriptional knock out factor (TKO) which negatively regulates signal activation by type I interferons (IFN-α/β); and (2) analysis of prolactin (PRL) growth factor effects on the antiproliferative signal generated by IFN-α/β-induced STAT1. Progress: (1) Amino acid (AA) sequences were obtained from multiple fragments of the TKO polypeptide (~20 kDa) isolated by 2D PAGE and subjected to tryptic digestion. Sequence homology showed similarity to a widely distributed group of 198 AA proteins present in eukaryotes and prokaryotes. Several nucleic acid sequences are highly conserved. RT-PCR and immunoblots with antibodies to two peptide fragments indicated expression of TKO or homologous protein in several breast cancer cell lines. Current work is assessing the relationship biologic of TKO activity to specific molecular forms or mutations of the ancestral protein. (2) Analyses of PRL growth factor interactions with IFN-α/β in differentiated (T47D, MCF-7) or undifferentiated (BT-20) breast cancer cells verified that STAT1 phosphorylation by PRL and IFN-α/β is non-competitive, so that endogenous PRL production would not constrain the antiproliferative effects of IFN-α/β. Phosphorylations of STAT3 and STAT5 by PRL were independent of IFN-α/β, but IFN-γ enhanced these signals by an uncertain mechanism.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	24
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS

(5) INTRODUCTION

Interferon therapy in breast cancer: Chemotherapy with conventional genotoxic agents places women with breast cancer at an increased risk for development of secondary leukemia[1]. Treatment decisions for young woman (< age 50 years) with stage II or breast cancer thus can pose a difficult dilemma for oncologists, particularly when the breast cancer is negative for estrogen receptors. Interferons (IFNs) are natural cytokines with antiproliferative properties. Type I interferons (IFN-α/β) block entry of responsive cancer cells into S-phase of the cell-division cycle[2,3] and act to induce cell differentiation or G_0 status[3,4]. Potential benefits of IFN treatment include mitigation of cytotoxic side-effects, and avoidance of permanent damage to normal non-proliferating tissues, with elimination of the hazard of delayed carcinogenicity. IFN-α/β has been tested repeatedly in preclinical models[5-8] and in phase I/II clinical trials[6,9-11]. Currently IFN-α is included as adjuvant therapy in some treatment protocols[11].

Stat protein phosphorylation mediates interferon (IFN) actions: Expression of genes leading to the principal biologic actions of IFN-α/β, including antiproliferative activity, depends upon the transcriptional function of cytoplasmic Stat proteins which serve in a dual capacity as signal transducers and activators or transcription[12-14]. In the case of IFN-α/β, signal transduction is initiated by dimerization of type I specific IFN receptors (IFNAR)[15] which are tyrosine phosphorylated by and non-covalently associated with reciprocally interdependent Janus tyrosine kinases (Jak1 and Tyk2)[12-16]. Sequential tyrosine phosphorylations of Stat2 and Stat1 proteins[17,18] precede their association as heterodimers and nuclear translocation[12-14,19]. The phosphorylated translocated Stats bind to highly conserved promoter regions upstream of IFN stimulated genes[20], and thus enhance or initiate transcription. Relevant nucleotide elements within upstream promoter regions include a gamma activated sequence (GAS) of IRF-1 GAS[18,20,21] and an $F_c\gamma$ element within the gamma response regions (GRR)[20,22,23]. These bind Stat1 homodimers. Another element, the interferon sensitive response element (ISRE), uniquely binds ISGF3 which is a multimeric complex of Stat2-Stat1 (ISGF3α)[13,18,19,24] that incorporates a 48 kDa DNA-binding protein (ISGF3γ)[24]. The ISRE acts as a positive transcriptional regulator in a large proportion of known interferon responsive genes[20]. The intranuclear translocation of ISGF3 and maintenance of gene transactivation by IFN directly correlates with the Stat phosphorylation status[19].

Activation of Stat1 through receptors for IFN-α/β depends upon the prior tyrosine phosphorylation (activation) of Stat2[17]; however Stat2 activation is not required for Stat1 activation in association with receptors of IFN-γ, prolactin or other agents activating the Jak-Stat pathway[12,18]. Thus, IFN-α/β can not generate a Stat1 signal in mutant cells which lack either Stat2 or the Janus kinases Jak1 or Tyk2[12,25]. *In vivo*, deficiencies of Stat1 tyrosine phosphorylation have been linked to a loss of IFN stimulated antimicrobial defenses[26, 27]; and a physiologic specificity of Stat1 proteins in the biologic responses of mice to IFN was clearly shown by targeted disruption of the Stat1 gene[27]

TKO as an inhibitor of IFN-α/β signals mediated by Stat1: Present investigators discovered that the signal transduction pathway for IFN-α could be selectively interrupted by a negative regulator of transcriptional activation which is detected in the cytoplasm of transformed cells[28]. This novel negative regulator was characterized as a polypeptide with a molecular weight of ~20 kDa and tentatively designated "transcriptional knock out factor" or "TKO". The mechanism of TKO negative regulatory action was related to inhibition of DNA binding by the 48 kDa-ISGF3γ subunit of the multimeric ISGF3 transcriptional holocomplex[28]. The 48 kDa-ISGF3γ polypeptide is structurally homologous to a family of IFN regulatory factors (IRF) which include a DNA-binding tumor suppressor protein IRF-1, an oncoprotein IRF-2, and a gene repressor ICSBP[21,24]. They are members of the *myb* family involved both in cell proliferation and apoptosis[13,21,24].

Potential for cross-talk in the signal pathways of IFN and prolactin (PRL): In addition to the families of interferon and interleukin cytokines, certain polypeptide hormones or growth factors, including erythropoietin, PDGF, growth hormone, and prolactin (PRL) activate gene expression through the Janus kinase / Stat protein pathway[12,13,23,29-31]. PRL is a principal mammotrophic growth factor, which stimulates physiologic proliferation of mammary epithelium prior to lactation. Intriguingly, PRL can serve both as a mammary epithelial growth promoter, and as a lactogenic differentiation agent [32-35]. Up to 70% of human breast cancers have proven positive for PRL receptors[33]; several common cell lines derived from breast cancers express abundant PRL receptors and some can proliferate in response to PRL *in vitro*[33,34]. In rodents, excess PRL can initiate mammary gland nodular hyperplasia and neoplastic transformation[32]. Although PRL classically originates from pituitary gland acidophil cells, recent work indicates that it can be endogenously produced by human breast cancer cells[36,37].

Like the type I IFNs, PRL induces phosphorylation of Stat proteins 1, 3 and 5[38]. Thus at the level of signal transduction mediated by Stat1-phosphorylation, the therapeutic antiproliferative action of IFN-α could in principal be modulated competitively by endogenous tumor-secreted PRL acting through an autocrine/paracrine feedback loop[36,39].

Purpose of project: The current project extends two sets of observations: (1) Original work from our laboratories which showed that a cytoplasmic extract from transformed cells (TKO) interrupts the Stat1 signal transduction pathway of IFN-α/β[28]; and (2) published work of others[36,37] showing that human breast cancer cells can secrete PRL and thus might autonomously activate Stat1 by tyrosine phosphorylation.

Scope of research:

(1) Major effort has involved characterization of TKO. Technical approaches have included purification of a protein with TKO activity from cancer cell extracts, AA sequencing, database analyses for matching cDNAs, RT-PCR of breast cancer cells to detect closely related cDNA sequences reflecting presumptive mRNA structure, primary extension sequencing of a TKO-candidate cDNA, cloning of a candidate cDNA and expression in bacteria or baculovirus systems to produce immunogenic protein.

(2) A second direction of work has explored for any negative cross-talk of PRL with the antiproliferative signal generated by IFN-α/β. Technical approaches have included immunoprecipitation and Western blot analyses of Jak and Stat proteins extracted from appropriately treated lines of human breast cancer cells, electrophoretic mobility shift assays (EMSA), auxiliary EMSA to test supershifting of Stat-DNA complexes, and conventional studies of cell proliferation parameters by cell counts, [^{3}H]-thymidine (dThd) uptake or MTT colorimetric assays.

Prior accomplishments during this project:

Detection of TKO action in breast cancer: We detected TKO activity in breast and cervical cancer cell extracts by means of EMSA using specific oligonucleotide probes to represent the ISRE sequence[28] of an IFN- stimulated gene (ISG54K). Although both Stat2-Stat1 heterodimers and the p48-ISGF3γ each individually are capable of binding to an ISRE nucleotide sequence, the binding affinity is ~25-fold increased when subunits are combined in the ISGF3 holocomplex[24]. By competing with the p48-ISGF3γ, TKO functionally antagonizes its binding to a [^{32}P]-oligonucleotide probe. In EMSA the TKO inhibition thus results either in absence of a band shift or a band mobility "supershift". This can be detected either with a [^{32}-P]-ISRE probe or IRF-1 GAS probe[28], and proved significant in cytoplasmic extracts from a line of human cells (Zr75-1) originating from breast cancer[40] (Fig. 2, 1995 Progress Report).

Purification of TKO for AA microsequence analysis: A line of cervical cancer cells (C33A) proved optimal for isolation of TKO, because these cells grow rapidly in mass suspension cultures. Using extraction procedures refined during this project (1995 Progress Report), ~300 ng of highly purified product was obtained from each of several batches of ~70 liters of C33A cells grown in mass spinner cultures at ~1 x 10^6 cells/ml. The protein functional quality ("specific activity") was verified by EMSA using recombinant p48-ISGF3γ, and 8 potentially useful batches were prepared.

Pre-analytic purification of protein with TKO activity was refined under the direction of project collaborator Dr. E. Petricoin. This involved dounce homogenization, separation of crude cytoplasmic extract, loading of membrane-free supernatant over heparin-sepharose resin, collection and pooling of flow-through, reloading onto a hydroxylapatite column and elution of the bioactive fraction with a sodium phosphate gradient. After steps for depletion of non-specific proteins the bioactive material was concentrated on an hydroxylapatite column. Specific activity by EMSA assay thus was increased $>10^4$ X based upon relative protein concentrations in crude and purified extracts. Active fractions also were purified from extracts of the ZR 75-1 breast cancer cells, and a presumptive TKO polypeptide was resolved by two-dimensional gel electrophoresis (2D-gel). Fig. 3 in the 1995 Progress Report compared the electrophoretic mobility of TKO polypeptide(s) in the ZR-75-1 breast cancer cells and C33a cells in 2D-gels. The molecular weight was estimated at ~ 20 kDa. Specific bioactivity was confirmed by a depletion procedure in which purified product was exposed to a synthetic N-terminal peptide fragment (125 AA) of the p48-ISGF3γ ligand prior to 2D-gels.

Lack of biologically significant IFN-PRL cross-talk at the level of Jak/Stat signaling in human breast cancer: Three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) were tested for Stat activation by IFN-α/β or PRL. Results indicated that signalling might be cell dependent both at the levels of Janus kinase and Stat activation. IFN-α/β had an expected effect in activating Jak1 and Tyk2 protein kinases involved in Stat2-Stat1 tyrosine phosphorylations, similar to results with other cell types. Although previous work had shown that Jak2 was the kinase primarily involved in phosphorylation of Stat1 after PRL treatment, PRL also activated Jak1 and Tyk2 in the breast cancer cells. In the highly responsive T47D cells, Jak1, Jak2 and Tyk2 had comparable dose responses and activation kinetics (1996 Progress Report).

Despite known disparities in negative growth regulatory actions of IFN-α/β and positive growth regulation or cellular differentiation by PRL, no evidence of an antagonism between IFN-α/β and PRL signal pathways was detected at the level of the Stat2-Stat1 activation which is so crucial to IFN-α/β signal transduction and biological actions. Indeed Fig. 3 of the 1996 Progress Report showed an additive effect on IFN-α-induced Stat1-Stat2 tyrosine phosphorylation when T47D cells were treated with IFN-α + PRL or when MCF-7 cells were treated with IFN-β + PRL.

(6) BODY:

Functional activity of isolated TKO protein: Identity of the ~20 kDa protein isolated from purified ZR-75-1 or C33A cytoplasmic extracts was verified circumstantially by depletion analyses, in which the TKO activity was eliminated by exposure of highly purified cell extract to *in vitro* translated p48-ISGF3 bound to agarose. The reproducible protein spots resolved on 2D-PAGE (e.g. Fig. 3, 1995 Progress Report) also disappeared after this treatment.

TKO amino acid sequence analysis: Denatured TKO protein localized after 2D-PAGE with amido black stain, was transferred to nitrocellulose. After washes with HPLC grade water, the membrane was wet frozen at -70°C for trans-shipment in dry ice. Tryptic digests for mass spectrometry were prepared by Dr. Steven Gygi in the laboratory of Dr. Ruedi Aebersold in the Department of Microbiology at the University of Washington in Seattle. Preliminary database searches by peptide mass matching were followed by mass spectrometric microsequencing of multiple fragments: svdetlr; ighpapnfk; adegisfr; dislsdyk; tiaqdygvlk; lvqafqftdk; glfiiddk; qgglgpmniplvsdpk. Public data base searches showed a close match to sequences which are characteristic of ~22 kDa proteins with ~200 AA. These comprise a newly classified superfamily (AHPC/TSA) of alkyl hydroperoxidase reductase C / thiol-specific antioxidants[41]. Proteins in this superfamily exhibit a number of highly conserved AA sequences including glfiiddk, etlr, or qafqf which were found in our samples and in some human and murine genes including OSF3, MSP23 and human NKEF[42-45]. Conserved sequences also may be found in proteins of some yeasts or prokaryotes[43].

RT-PCR amplification of breast cancer cell mRNA from TKO-related genes: Based upon cDNA sequences for known human genes in the AHPC/TSA family[43-45], we prepared sense (5'-GGTGTCTGGTTAGTTTCTGC-3') and antisense (5'-GACGGGATAGTGACTTTCGTTAC-3') primers for RT-PCR to amplify cDNA representing a 195 base pair (bp) conserved region (bp 33 → 228) of the processed mRNA deduced to be expressed by the TKO-related human NKEF gene and closely related OSF3 and MSP23 murine genes. The amplified region encompassed codons for two of the microsequenced tryptic digest fragments (ighpapnfk and dislsdyk), and which are well conserved. A minus strand probe was designed to detect a conserved intervening sequence 5'-TGTTATGCCAGATGGTCAGT-3'(~vmpdgq).

With RNA samples extracted from the ZR-75-1 breast cancer line previously shown to exhibit TKO function (1995 Progress Report), RT-PCR amplified a cDNA product of expected molecular size (Fig. 1 top). This PCR product was generated by just 18 cycles of amplification and sharply resolved by DNA gel electrophoresis (ethidium bromide stain). It was compared to equivalently amplified hypoxanthine phosphoribosyl transferase (HPRT) as a human "housekeeping" gene RNA control (Fig. 1, middle). Fig. 1, bottom, shows that using the minus strand cDNA probe for quantitation of RT-PCR product, TKO-related gene(s) were strongly expressed in four breast cancer cell lines: MCF-7, ZR-75-1, T-47D and Hs578T. The gene(s) also appeared to be expressed in C33A cervical cancer cells and low passage foreskin fibroblasts. In general, expression appeared to be slightly greater in confluent cultures (+) as compared to subconfluent cultures (-). Further work now is necessary to determine whether the presumptive sequence of TKO gene amplified from these cancer cells is mutated when compared to presumed wild type sequence(s) (see below).

Immunoblot evidence for expression of TKO-related protein in breast cancer cells: Two peptide sequences, selected based upon the full cDNA sequence of presumptively related proteins in the AHPC/TSA family, were synthesized and used to elicit rabbit antibodies. One of the synthetic sequences encompassed two adjacent peptide fragments microsequenced by Dr. Gygi: tiaqdygvlk and adegisfr (beginning at position 111 of the NKEF coding sequence). The other amino acid sequence selected was the C-terminal kpgsdtikpdvqkskeyfskqk which is well conserved in mammalian AHPC/TSA genes[43]. Fig. 2 shows results with whole cell SDS extracts resolved by SDS-PAGE (12%) and subjected to immunoblotting with polyclonal rabbit antibodies to the "111" peptide and "C-terminal" peptides. With two breast cancer cell lines (MCF-7 and ZR-75-1), and the cervical cancer C33A cell line, each antibody detected a chemiluminescent band of ~22 kDa consistent with the expected ~200 AA polypeptide (derived value from cDNA codons). The antibody to "111" peptide also localized at higher molecular weight positions. Since the proteins were denatured in hot 1% SDS either with β-mercaptoethanol or dithiothreitol, results suggest the presence of larger precursor proteins. Further experiments with peptide competitor or with affinity purified antibodies to an entire TKO-like protein or to larger subunits should resolve these questions.

Preliminary tests of anti-peptide antibodies on TKO-activity in EMSA: The polyclonal antibodies to peptide fragments described above also were utilized in an effort to deplete TKO activity from cancer cell extracts prior to EMSA with P48-ISGF3γ (using a standard [^{32}P]-Fcγ-GRR probe). No depletion of DNA-binding was noted. Since the rabbit antibody had been prepared against a linearized polypeptide rather than a native TKO-protein, such a result may simply indicate that tertiary protein configuration is critical to ligand binding. The next effort; therefore, was to express a complete human macromolecule of the AHPC/TSA family using recombinant DNA technology. The ultimate objective is to stimulate antibodies to epitopes in native configuration. Presumably many of these epitopes would be shared with the TKO protein.

Expression of recombinant TKO-related proteins: In order to expedite biosynthesis of an appropriate TKO immunogen, we deployed two complementary strategies.

(1) *GST-tagged protein:* Project collaborator S. Vande Pol used the NKEF gene sequence for expression of TKO ligated to a glutathione S-transferase (GST) tag. The primers for amplification of cDNA were modified to include 5'-EcoR1 and XhoI-3' restriction enzyme cleavage sites. The full coding sequence was cloned into a pPCR-Script (Stratagene) vector, and amplified in E. Coli. The insert was excised with and subcloned in the pGEX-4T-1 expression vector (Pharmacia) in frame for N-terminal expression with GST. The recombinant plasmid was used to transform of E. Coli DH 5α. After bacterial lysis, clarified supernatant was affinity purified with glutathione sepharose 4B for inoculation into rabbits. Preliminary tests of this antibody are being initiated by collaborator A. Larner, both for Western blots of denatured cells and EMSA assays using native C33A extract.

An anticipated drawback is that GST is a 27 kDa protein which can modify antigenicity when ligated to another protein such as TKO. Although it can be cleaved free with a site-specific protease, this is a complex procedure of limited success. Therefore, we have initiated production of a polyhistidine (H)-tagged candidate protein using a Baculovirus Expression System (GIBCO BRL). Since the H tag is of relatively low molecular weight, the epitope would be less likely to alter TKO immunogenicity.

(2) *His-tagged protein:* As in plan (1) above, the primers for cDNA amplification were modified to include 5'-EcoRI and XhoI-3' restriction enzyme cleavage sites. RT-PCR product from C33A or ZR-75-1 cells was cloned into pPCR-Script and amplified in transformed *E.Coli* (DH 5α). Several colonies were streaked and screened with an appropriate [^{32}P]-cDNA internal probe to verify insertion of the presumptive TKO coding sequence. The insert was excised and subcloned into pFastBac HT donor plasmid (GIBCO method). DNA mini-preps and restriction endonuclease digestion verified correct insert orientation and the presence of H-tag in frame at the N-terminus. Commercial bacteria (*E.Coli*, Bac 10) previously transformed with baculovirus (Bacmid) and containing a disrupted *Lac7 gene* were transformed with the donor plasmid. A miniprep of high molecular DNA from this step currently is being sequenced by PCR using

fluorescent labelled dye terminators (Perkin Elmer Applied Biosystem 377 A) for confirmation. Antibody identification of H-tag or color identification of Lac7 disrupted (white) colonies (GIBCO protocol) containing the Bacmid with transposed DNA insert will allow clone selection for the final biologic amplification of candidate TKO cDNA in SF9 insect cells. A mini-prep of high molecular DNA will be used for transfection of the insect cells with CellFectin reagent. Released baculovirus with the H-tagged DNA insert then will be used at low multiplicity of infection and be plaque purified by infection of SF9 cells. Once optimal conditions for SF9 cell infection have been resolved, the H-tag recombinant will be mass expressed for use as an immunogen and in direct testing with EMSA. Separation of H-tag protein from clarified lysates of infected insect cells should be cleaner than the extracts of GST-fusion protein from transformed bacteria which are difficult to rid of cell wall materials. H-tagged molecules can be affinity purified by passage through a HiTrap column (Pharmacia) with Ni^{2+} chelated to the gel matrix.

Testing of TKO antibody: In addition to direct testing of recombinant TKO-like protein by EMSA with appropriate DNA probes, the initial plan is to test for a functional antagonism of TKO from breast cancer cells by means of polyclonal antibodies to H-tag candidate TKO protein. Pre-immune and immune rabbit sera will be screened by EMSA either for functional activity in neutralizing or supershifting the complex of p48-ISGF3γ with a [^{32}P]-GRR or [^{32}P]-ISRE probe. Any samples yielding a positive reaction will be tested further by indirect or direct immunofluorescence with fresh or fixed TKO-positive breast cancer cells from the ZR75-1 line and C33 positive control cells[41]. Depending upon characteristics and specificity of the antibodies, more extensive tests with control transformed and non-transformed cell lines of various types, samples of fresh or frozen biopsy tissues, samples of deparaffinized surgical biopsies, or aspiration cytology samples can be undertaken as proposed in the original statement of work. If appropriate, production of monospecific antibodies by intranodal or intraperitoneal mouse injections will follow. Each antibody also will be tested for potential use in Western blots.

Signal specificity of IFN and PRL shown by formation of independent and qualitatively different Stat-DNA complexes: Despite significant overlap in the activation of Stat proteins by type I IFNs and PRL, previously documented in this project, an important question remained as to whether PRL might interfere with IFN-induced Stat signals at the level of DNA-binding required for transcriptional activation. The formation of Stat-DNA complexes was analyzed by electrophoretic mobility shift assays using probes derived from several established IFN-responsive genes. Since T47D cells express a relatively high number of PRL receptors per cell (~ 20 x 10^3), and exhibited the strongest responses to combined PRL and IFN-β, nuclear extracts from these cells were chosen to examine inducible Stat binding to [^{32}P]-oligonucleotide probes representing the ISRE of the ISG15 gene, the IRF1 GAS promoter element and the $F_c\gamma R$ GRR element. EMSA disclosed significant differences in formation of the IFNβ and PRL-induced complexes, and showed that at the level of DNA-binding co-stimulation with PRL did not alter signals induced by IFN-β.

As anticipated, Fig. 3, panel A, shows that only IFN induced formation of a DNA-binding complex with the ISRE enhancer element and that this complex was completely supershifted or neutralized by antibody against either the Stat1 or Stat2 components of the ISGF3 heterodimer

know to bind to the ISRE. Although IRF-1 is a tumor suppressor gene activated both by IFNβ and PRL, the IRF1 GAS complexes with Stats differed. PRL induced two separate complexes, a major slow migrating complex and a minor fast-migrating complex (Fig. 3, panel B, lane b); whereas IFN-β induced a single and stronger fast-migrating complex (lane c). Both fast-migrating complexes were clearly supershifted with anti-Stat1 (lanes e,f). The slow-migrating complex was not shifted by anti-Stats 1,2,3 (shown) or 5a (not shown) but may reflect a complex with Stat5b[38]. Results with the GRR probe generally proved similar, except that PRL induced only the slow-migrating complex which could not be supershifted with antibodies tested (Fig. 3, panel C). Additional evidence for independence of PRL and IFN signals was provided by differences in the DNA-binding complexes induced with the [^{32}P]-GAS sequence of the β-casein gene promoter (Fig. 3, panel D). The β-casein gene is a known Stat5-regulated gene and the β-casein-derived GAS formed two distinct PRL-induced complexes which corresponded to two PRL-inducible and Stat5 positive complexes observed in rat Nb2 thymocytes[38]. These DNA complexes also were not supershifted by Stat5 antisera.

PRL fails to modulate the antiproliferative effect of IFN-β: Biologic autonomy of the IFN and PRL effects evidently was maintained despite functional sharing of the critical Stat1 signal component. When T47D cells in mid-log growth were treated with IFNβ and/or PRL as shown in Fig. 4, the decrease in uptake of [^{3}H]-dThd caused by IFN-β was not diminished. Similarly, no change was observed in a parallel set of experiments where the cells with pretreated with IFN-γ. These results were complemented by an MTT assay for metabolically viable cell mass. An important result of clinical interest was the finding that IFN-γ pretreatment consistently enhanced the action of a type I IFN. While not a unique finding, this additive effect of type I and γ-IFNs has often been overlooked in design of therapeutic protocols.

Independence of MAP kinase activation by PRL: The Erk MAP kinases have been implicated as critical regulators of Stat1 and Stat3 functional activation by serine phosphorylation[46]. Both PRL and type I IFNs can activate this pathway through *raf* as mediator. The possibility of crosstalk between PRL and IFN pathways at the MAP kinase level was therefore examined. Antibodies specific for the ser/thr-phosphorylated forms of Erk-1/2 (Promega) were utilized for this analysis. Fig. 5 shows that despite marked Stat activation by type I IFNs in the breast cancer system, there was no overt stimulation of Erk-1/2 phosphorylation in any of the cell lines tested. In contrast, PRL activated these MAP kinases to a variable degree both in MCF-7 and T47D cells. The absence of an effect in the line of BT-20 breast cancer cells may reflect high constitutive phosphorylation. In sum, there was no evidence of PRL and IFN cross-talk and evidently no specific PRL modulation of IFN Stat functions related to Stat serine phosphorylations which may depend upon the Erk-MAP kinase pathway. IFN-γ also exerted no significant effect at this level.

TEXT FIGURES

Fig. 1 RT-PCR for amplification of a TKO-like cDNA from human breast cancer RNA. RNA was extracted from fresh cell monolayers with Trizol (1 ml/10 cm^2 of dish). After phase separation with phenol-chloroform and precipitation with isopropyl alcohol, RNA pellets were washed with 75% ethanol and vacuum dried. Resolubilized RNA was brought to a final concentration of 2 μg/32 μL of RNAse-free H_20, briefly heated to 65°C and chilled on ice for use in reverse transcription with antisense primer on first-strand reaction beads. The reverse strands were amplified using sense and antisense primers described in the text. In repeated cycles (15-30)/ PCR with AmpliTaq (Perkin-Elmer) was performed at 94°C for denaturation, 54.9°C for annealing (based upon design of the primer as determined by the MacVector program), and 72°C for extension. Completed samples were run in 2% agarose gel for ~1 hr, stained with ethidium bromide (1μL/ml) for 10 min and photographed in UV light. Top: The far left lane shows the DNA ladder molecular weight control (minimum 123 bp). Other lanes show product from ZR-75-1 RNA. Center: comparisons of "housekeeping" HPRT cDNA product from ZR-75-1 cells (left) and TKO-like cDNA (right). The HPRT cDNA is of smaller size, Bottom: Southern blot comparisons of cDNA product (18 amplification cycles) from RNA extracted from human fibroblasts (FS4), cervical cancer cells (C33A) and four breast cancer lines at confluent (+) or subclonfluent (-) stages of growth. The cDNA transfered to PDVP membrane was hybridized with a minus strand [^{32}P]-oligonucleotide probe (see text).

Fig. 2 Western blots of TKO-like protein in breast cancer cells. Protein was extracted with hot SDS-buffer from fresh monolayers of breast cancer cell lines (MCF-7, ZR75-1), cervical cancer cells (C33A) and serially passaged foreskin fibroblasts (FS4). After protein separation by SDS-PAGE (12%), proteins were transferred to PVDF membrane and reacted for 2 hours at room temperature with a 1:1000 dilution of the antibodies to "111" or "C-terminal" peptides as indicated (see text for descriptions). Antibody binding was visualized by enhanced chemiluminescence (ECL)

Fig. 3 Analysis of STAT-DNA complexes induced in breast cancer cells by IFN or PRL. Quiescent T47D cells were incubated with IFN-β (1000 IU/ml) or/and human PRL (20 nM) as indicated for 10 min at 37°C, and whole cell lysates were prepared for EMSA. Selected lysates, corresponding to 10 μg of protein, were further incubated either with normal rabbit serum (lanes a-d), or with characterized anti-Stat1 (lanes e,f), anti-Stat2 (lanes g,h), or anti-Stat3 antibodies (lanes i,j) then combined with 1 ng of [^{32}P]-labelled oligonucleotide probe corresponding either to: panel A, the ISRE of ISG15, panel B, the GAS response element of the IRF-1 gene promoter, panel C, the GRR response element of the $F_c\gamma$R1 gene, or panel D, the PRL response element of the rat β-casein gene.

Fig. 4 Non-competitive effect of PRL on IFN anti-proliferative activity in breast cancer cells. T47D cells in mid-log growth were treated with IFNβ (1000 IU/ml) and/or PRL (10 nM) as indicated. In a parallel set of experiments the cells with pretreated with IFN-γ. After 24 hr, cells were treated as indicated: (A) Cells were pulse-labelled with [^{3}H]-dThd (10 μCi/ml) for 4 hours, harvested onto fiberglass filters, fixed, and washed with graded ethanol solutions. Radioactivity (cpm) was quantitated by liquid scintillation counting of the dried filters. (B) Cell viability at 24 hours was determined by MTT assay. The bars marked with an asterisk* indicate statistically significant data ($p < 0.05$).

Fig. 5 PRL stimulation of MAP kinase (Erk-1/2) phosphorylations independent of IFN-β signalling. In one set of experiments, quiescent breast cancer cells were incubated for 15 min at 37°C with PRL (20 nM) and/or IFNβ (1000 U/ml) as indicated for lanes b-d. In another set of experiments (lanes e-h), the same cells and a control (lane e) were pretreated for 24 h with IFNγ (10 nM/ml). Whole cell lysates were prepared in SDS; proteins (50 μg/lane) resolved by SDS-PAGE (12 %) subsequently were transferred to PVDF membrane. Membranes were incubated with rabbit antibody specific for the phosphorylated-activated forms of Erk-1/2 MAP kinases (Promega, Cat. no. V667A). Antibody binding was localized by enhanced chemiluminescence (ECL). Note that IFN-γ appeared to increase the effect of PRL in MCF-7 cells (lanes f and h).

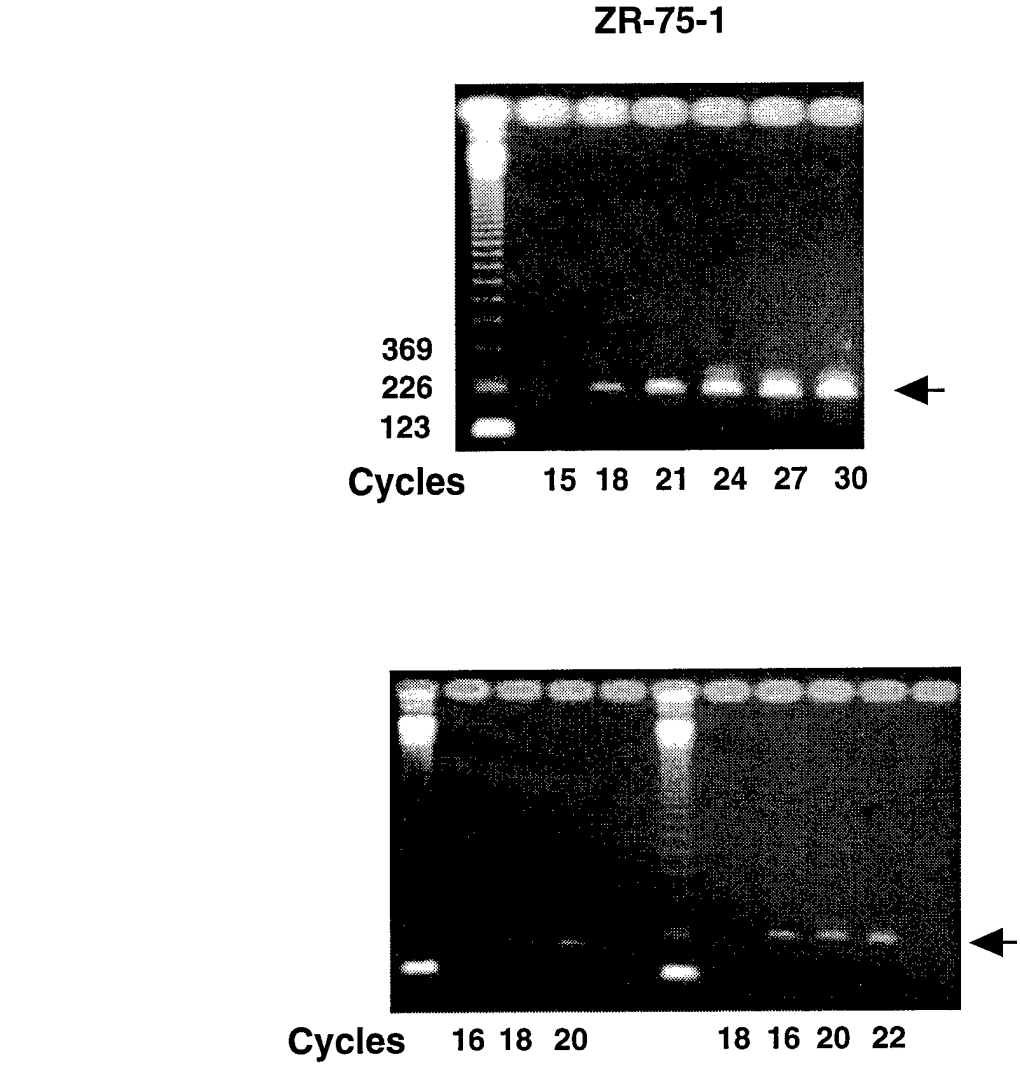

ZR-75-1
369
226
123
Cycles 15 18 21 24 27 30
Cycles 16 18 20 18 16 20 22
Cell line Fs4 C33a MCF-7 T-47D ZR-75-1 Hs578T
Confluence + − + − + − + − + − + −

Figure 1

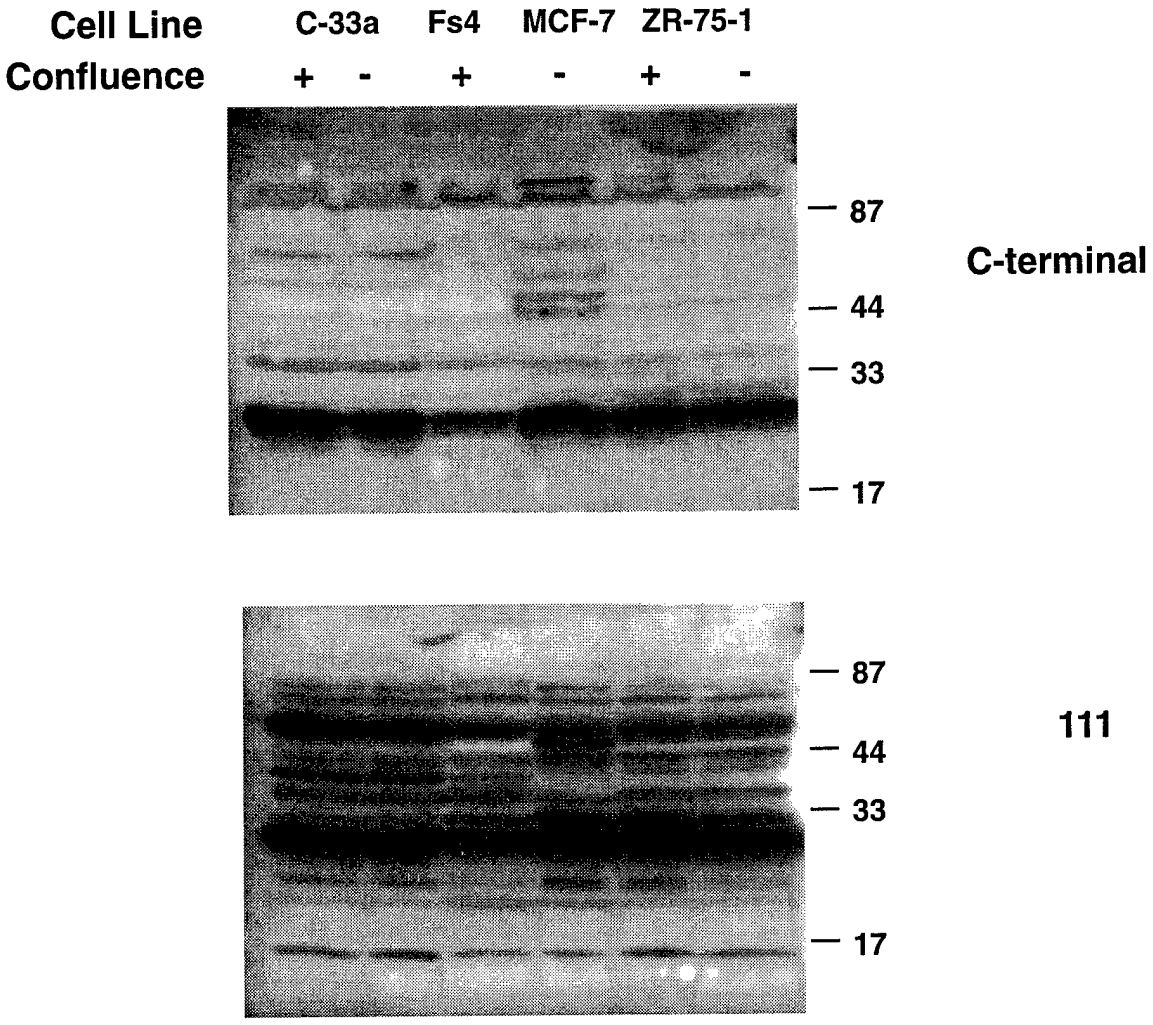

Cell Line
C-33a
Fs4
MCF-7
ZR-75-1
Confluence
+ - + - + -
87
44
33
17
C-terminal
87
44
33
17
111

Figure 2

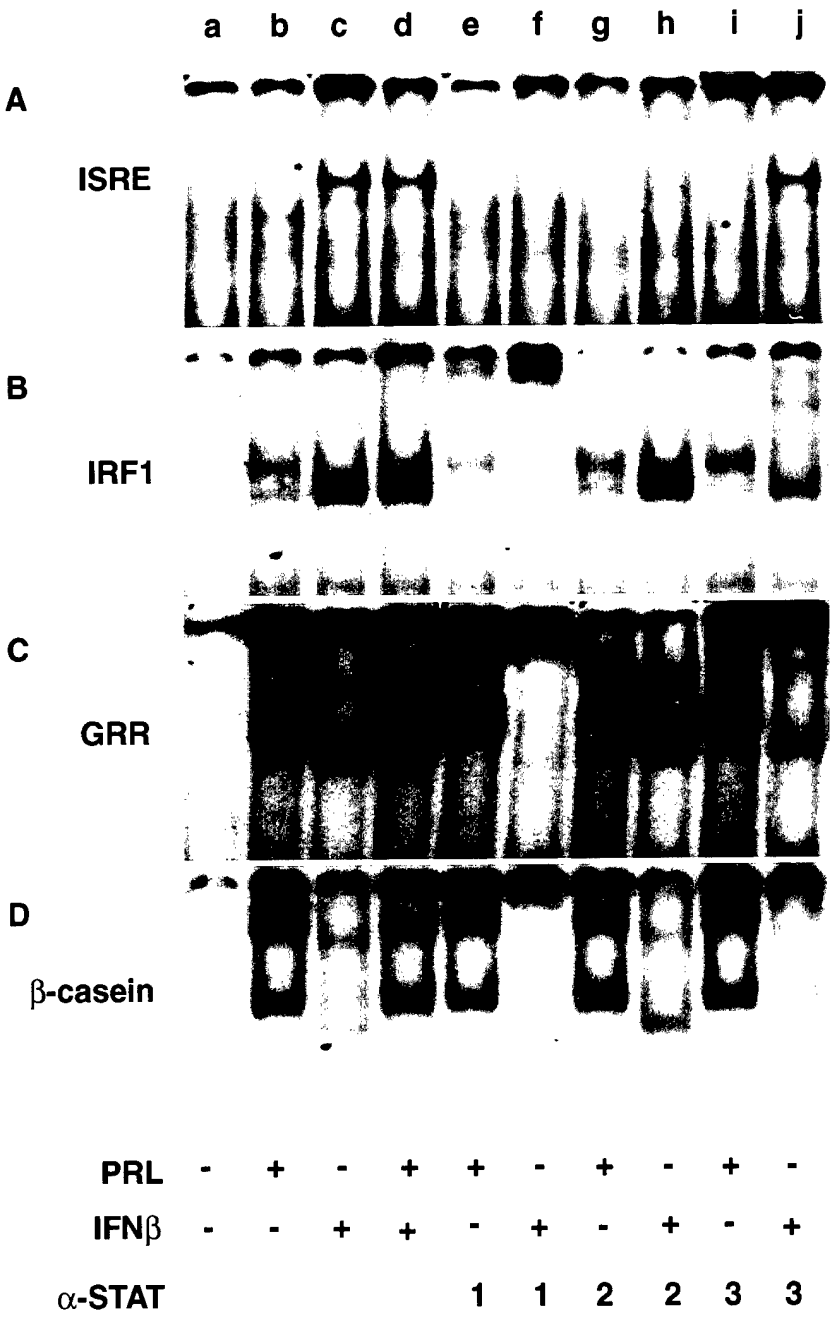

a b c d e f g h i j
A
ISRE
B
IRF1
C
GRR
D
β-casein
PRL - + - + + - + - + -
IFNβ - - + + - + - + - +
α-STAT 1 1 2 2 3 3

Figure 3

Fig. 4

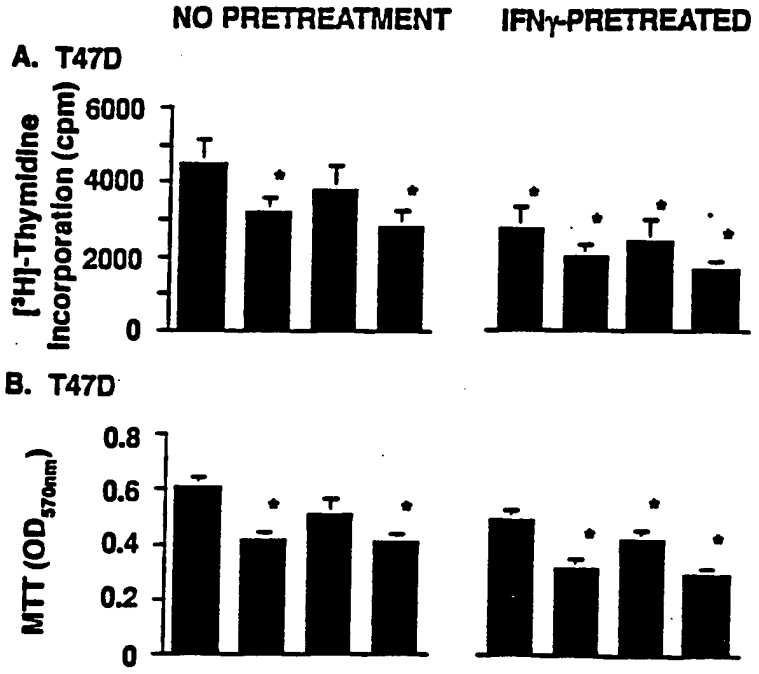
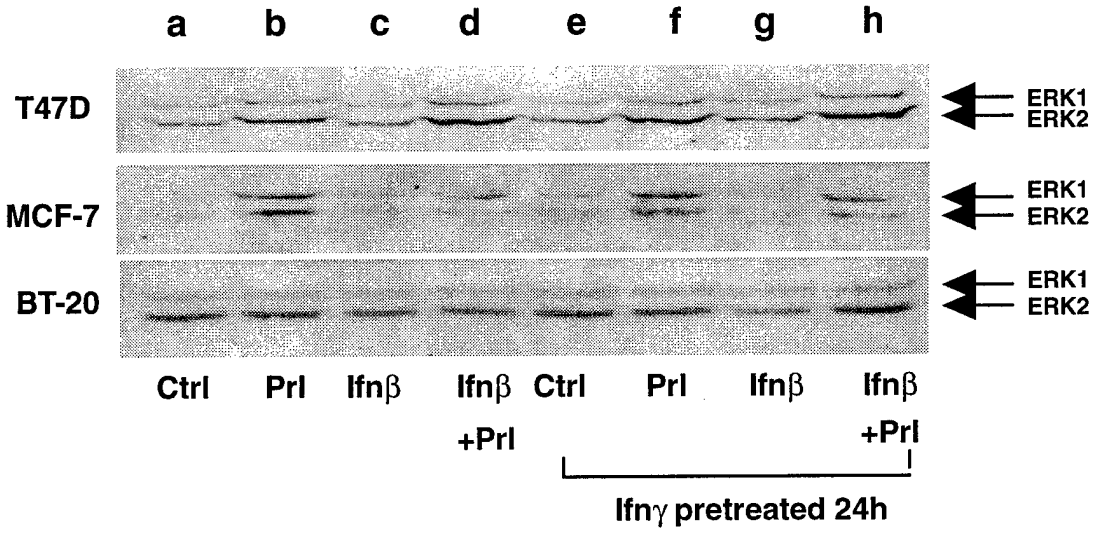

NO PRETREATMENT
IFNγ-PRETREATED
A. T47D
[³H]-Thymidine Incorporation (cpm)
6000
4000
2000
0
B. T47D
MTT (OD570nm)
0.8
0.6
0.4
0.2
0

Fig. 5


a
b
c
d
e
f
g
h
T47D
MCF-7
BT-20
ERK1
ERK2
Ctrl
Prl
Ifnβ
Ifnβ +Prl
Ctrl
Prl
Ifnβ
Ifnβ +Prl
Ifnγ pretreated 24h

(7) CONCLUSIONS:

TKO function in breast cancers: A novel cytoplasmic protein, which can negatively regulate transcriptional activation by type I IFNs occurs in human breast cancer cells and corresponds to an ~20 kDa polypeptide purified from human cervical carcinoma cells (C33A) and designated "TKO" (transcriptional knock-out factor). Tryptic digestion and AA microsequence analysis of multiple peptide fragments from purified protein with high specific TKO activity has been accomplished in collaboration with Dr. Steven Gygi in the laboratory of Dr. Ruedi Aebersold, at the University of Washington in Seattle. Public database searches place this putative TKO protein within a group of 200 AA polypeptides containing several highly conserved and evolutionarily ancient coding sequences. These polypeptides recently have been classified with a superfamily of alkyl hydroperoxidase reductase C/ thiol-specific antioxidants (AHPC/TSA family). Based upon the cDNA coding sequences of two previously analyzed sequences of human genes, including NKEF, mRNA expression has been shown by RT-PCR in several breast cancer and other cell lines. More detailed microsequence comparisons by the method of primer extension and fluorescent labelling will enable us to determine whether the putative TKO sequence includes any oncogenic mutation(s). At the same time, we are in the process of amplifying a TKO-like candidate cDNA in the baculovirus system to achieve the objective of producing native protein. This is critical for generating polyclonal or monoclonal antibodies that can be utilized for immunoprecipitation and functional EMSA. Rabbit antibodies to selected peptide fragments already have been prepared. While these were not functional; they identified a common protein sequence in Western blots of several cell lines including breast cancers. A GST-fusion protein has been prepared and is currently being tested. With production of a recombinant H-tag fusion protein, we expect it will be feasible to generate and pilot test appropriate monoclonal or polyclonal antibodies per original statement of work and begin to evaluate the significance of protein expression in clinically relevant materials.

Non-competitive roles of PRL and IFN in Jak-Stat signal transduction and growth inhibition: Type I IFNs inhibit the growth of mammary epithelial cells and serve as adjuvant agents in breast cancer therapy. Conversely PRL is one of the principal mammotrophic factors and a tumor promoter in rodent mammary gland. Despite these contrasts in biologic impact, type I IFNs and PRL signals are mediated by some common JAK tyrosine kinases and STAT transcription factors. In addition, mitogen-activated protein kinases (MAPK) have been implicated both in IFN and PRL signaling. Since IFNγ is known to enhance IFN α/β responses in many cells, the effect of pretreatment with IFN-γ for 24 h on IFN α/β and PRL-regulated growth and signal transduction also was tested. Three representative human breast carcinoma lines, (T47D, MCF-7 and BT-20) were chosen for testing of signal induction by PRL and IFN-α/β. In all cells, IFN-α/β activated transcription factors Stats 2 and 1, whereas prolactin activated Stats 1, 3 and 5, but not STAT2. Combined effects of IFNα/β and PRL were consistently additive and revealed no competition for Stat1. Results were consistent with a role of Stat2 as a docking factor for Stat1 in relation to the IFNAR[17], and not necessary for action of the PRL receptor.

Recent evidence points to Stat5 as an anti-apoptotic factor, as well as a differentiation factor during mammary development[47]. Interestingly, it was activated by PRL, but not but not by IFN-α/β. While the MAP kinases have an established role in promoting cell growth and oncogenesis, induction of Erk-1/2 by PRL failed to counteract the growth-inhibitory effect of IFNs when tested in T47D and MCF-7 cells. Paradoxically, although Stat3 has been implicated in hematopoietic cell growth inhibition[12], it was activated by PRL rather than by IFN-α/β. Obviously there are cell-dependent differences in the roles of Stat proteins as reviewed just recently by Darnell[12]. Present work nevertheless seems to indicate that endogenous local or systemic production of PRL in patients with breast cancers would not be a significant factor in modulating the therapeutic actions of exogenously administered type I IFNs.

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