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13. ABSTRACT (Maximum 200) Elucidating the mechanisms by which hormones affect normal programs of differentiation and development in the breast is essential for understanding the complex role that hormones play in the pathogenesis of breast cancer. Studies of differentiation, development, and carcinogenesis in a variety of biological systems have emphasized the importance of protein kinases in these processes. We have identified two novel serine/threonine kinases; I43, a member of the CaM kinase family, and A32, a SNF1-related kinase. Expression analysis of I43 suggests a role late in pregnancy, possibly during differentiation since expression levels peak late in pregnancy and are also induced during NGF-treatment of PC-12 cells. Conversely, A32 levels peak early in pregnancy and decrease when PC-12 cells are treated with NGF suggesting a role early in differentiation. These kinases are also expressed in a mutually exclusive manner in mammary tumor cell lines derived from different transgenic animals. In order to determine the functional role of these kinases, we have begun to overexpress full-length clones in a variety of tissue culture systems and in transgenic mice which overexpress the kinase of interest in a mammary-specific fashion. As such, we believe these studies will contribute to our understanding of the role played by I43 and A32 in mammary gland development and carcinogenesis.				
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Heather Long Jordan 10/11/97
PI - Signature Date

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INTRODUCTION

Numerous epidemiologic studies have shown that breast cancer risk is strongly influenced by endocrine history. Endocrine history, in turn, determines the timing, course and extent of mammary gland development. This suggests that mammary gland carcinogenesis and mammary gland development are fundamentally linked. As such, elucidating the mechanisms by which hormones affect normal programs of differentiation and development in the breast is essential for understanding the complex role which hormones play in the pathogenesis of breast cancer. Studies of differentiation, development, and carcinogenesis in a variety of biological systems have underscored the importance of protein kinases in these processes. For example, *lck* is a nonreceptor tyrosine kinase primarily expressed in T-cell lineages, which associates with CD4 and CD8, and which is involved in translocations in childhood acute lymphoblastic leukemia [1]. Mice which are homozygous for the targeted disruption of *lck* by homologous recombination manifest arrested T-cell development prior to the CD4/CD8 double-positive stage[2]. The finding demonstrates that *lck* is required in T-cell ontogeny exemplifies the fundamental relationship between development and carcinogenesis. Therefore, we have initiated a systematic study of the role of protein kinases in mammary gland development and carcinogenesis. From this study, three novel kinases have been discovered that contain highly conserved amino acids and share regions of homology with other known kinases. We propose further study of these novel kinases to elucidate their potential function in breast development and carcinogenesis.

Specific Aim 1. Characterize the structure of novel protein kinase full-length clones. We have constructed and screened a PCR-derived cDNA library containing fragments of protein kinase catalytic domains expressed in the murine breast during development, and in cell lines derived from the normal, hyperplastic, or transformed mammary epithelium of transgenic mice expressing the *neu*, *myc*, *ras* or *int2* oncogenes. This screen has yielded 39 kinases, three of which appear to be novel. The analysis of conserved amino acid motifs in numerous kinases has emphasized that analysis of primary structure may reveal important clues regarding function in this class of proteins. Initial analysis of this type has allowed us to determine which protein kinase super-families these novel kinases are likely to belong to; I25 appears to be a receptor tyrosine kinases related to the *trk* family, I43 is most likely a Ca²⁺/calmodulin-dependent protein kinase (CaM-kinase), and A32 is a putative serine/threonine kinase without homology to any previously described kinase family. Full-length cDNA clones will be isolated and sequenced for each of these novel kinases. Putative functional domains and homologies will be determined by analysis of the full-length cDNA clones.

Specific Aim 2. Further characterization of the temporal and spatial expression patterns of kinases in the murine breast. Initial studies of I25, I43, and A32 have described their temporal pattern of RNA expression during mammary gland development and their expression in adult mouse tissues. These studies reveal distinct patterns of expression for each of these three protein kinases. Ribonuclease (RNase) protection analysis and Western blotting will be used to define: (a) the temporal pattern of expression during each stage of mammary gland development; (b) the regulation of expression by ovarian hormones. *In situ* hybridization will be used to determine the spatial pattern of kinase expression in order to identify those kinases which define biologically interesting subpopulations of epithelial cells, and in order to define changes in those subpopulations during development.

Specific Aim 3. Determine the role played by novel kinases in differentiation, proliferation, and carcinogenesis. Kinases have been shown to be critical for differentiation, development and carcinogenesis in a variety of biological systems. To examine the potential functional roles of the novel kinases, RNase protection and Western blot analysis will be used to define changes in expression levels as a function of differentiation and proliferation, in well characterized *in vitro* model systems. Kinases will then be overexpressed in these systems by retroviral-mediated transduction to determine the effect on the process of differentiation,

proliferation, and carcinogenesis. Differentiation and cell proliferation will be studied in mammary epithelial cells induced with lactogenic hormones, in neuronal cells induced with nerve growth factor (NGF), and C2C12 myoblasts induced by serum starvation. Mammary epithelial cells overexpressing each of the kinases will be assayed for changes in tumorigenicity as defined by focus formation, growth in soft agar, and the ability to form tumors following subcutaneous injection.

Transgenic mice will be used to study the impact of kinase overexpression on differentiation, proliferation, and carcinogenesis. The development and differentiation of the transgenic mammary gland will be assayed morphologically by analysis of whole mounts, and examination of hematoxylin and eosin stained sections to look for changes in ductal morphogenesis during puberty, alveolar formation during pregnancy, programmed cell death during postlactational regression, as well as hyperplasia, dysplasia, and carcinoma. In addition, the expression of defined molecular markers will be used to assay for subtle changes in programs of differentiation and development.

BODY

Note: Prior to completion of specific aim I, full-length sequence and functional data was published for novel kinase I25 [3], which suggests a role in neuro-muscular junction formation. In addition, further expression studies in the breast reveal low levels of I25 expression relative to other kinases and essentially no regulation at the RNA level. Therefore, we have focused our efforts on further characterization of the novel kinases, A32 and I43.

Technical Objective I: Isolate and characterize full-length cDNA clones for novel protein kinases.

Task I: Months 1-12: Isolate full-length cDNA clones for novel kinases.

In order to isolate full-length clones, cDNA libraries were generated using polyA RNA from a variety of sources. First strand cDNA was ligated into lambda phage arms containing bluescript plasmid flanking sequence (Stratagene) which allows for easy excision of clones following phage purification. Amplified libraries were plated such that 5×10^5 phage were screened at a time. Following duplicate filter lifts, ^{32}P -labeled probes were generated using the random primed labeling method and then hybridized to replica filters containing phage DNA. Positive plaques were cored, plated and rescreened until purified phage were isolated.

Using this library screening method, three independent clones for I43 were isolated from a mouse fetal brain library (Stratagene). All clones were approximately 1.5 kb which corresponds to the expected size for I43 as determined by Northern analysis. Since I43 appears to be a CaM kinase, the amino acid sequence for I43 was compared to other CaM kinases. The location of the putative initiation and stop codons for I43 are consistent with those from other CaM kinase family members. Given this information, we are confident that we have isolated a full-length cDNA clone for the novel serine/threonine kinase, I43.

The same approach was used in order to obtain a full-length clone for A32. Unfortunately, the large size, approximately 5 kb, and the lack of homology to other known kinases made this task much more difficult for A32. As such, multiple libraries were constructed and screened in order to isolate a full-length clone for this molecule. In addition, several other techniques, including 5' RACE (rapid amplification of cDNA ends) and inverse PCR, were employed in an attempt to isolate the 5' end of A32. A 4.9kb clone, designated "clone G", was isolated from a murine mammary tumor epithelial cell library. This cDNA contains a putative initiation methionine 30 bp from the 5' end, and open reading frame of xnts and a stop codon at nt 2175. Therefore, clone G contains a 30 bp 5' untranslated region (UTR) and a 3kb 3' UTR. In vitro transcription/translation of clone G using ^{35}S -labeled methionine results in a single product of the predicted size and confirms the presence of a Kozak consensus sequence surrounding the putative initiating AUG. Unfortunately, there are no stop sequences up-stream of this AUG which does not rule out the possibility of there being more A32 coding sequence upstream.

In order to determine if we had a full-length clone for A32, and to rule out the possibility that clone G contained a mutation due to the fact that it came from a tumor cell line library, an oligo dT primed cDNA library was constructed from polyA RNA isolated from day 14 mouse embryos. This library was screened using the 5' end of clone G in order to increase the chances of getting full-length clones. From this screen, 5 additional A32 clones were isolated, one of which (clone E8) was 30 bp longer than the original clone G. No up-stream stops were found in this clone either. However, the additional up-stream sequence allowed us to more rigorously compare this mouse sequence with human sequence obtained from screening a human fetal brain library. Conceptual translation of the two cDNAs reveals greater than 90% amino acid identity between mouse and human over the entire putative coding region for A32. Homology drops significantly to lower than 30% both 3' of the stop and 5' of the putative initiation codon. This high homology only in the coding region suggests that both the mouse and human clones are full-length.

Task 2: months 1-12: Characterize structure of protein kinases.

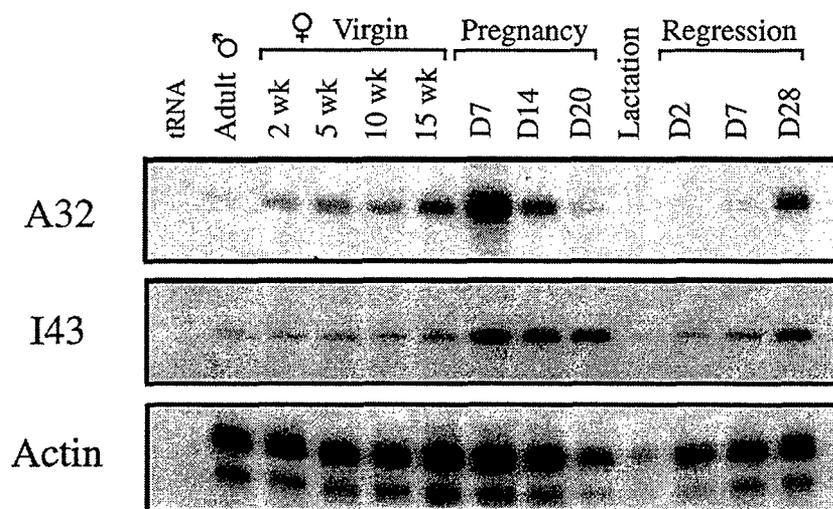
Analysis of amino acid sequence can be used to identify conserved motifs in proteins which may assist in determining protein function. Sequence analysis of I43 confirms homology to the CaM kinase family of serine/threonine kinases. Within the kinase domain, I43 is 70% identical to CaM kinase I and approximately 50% identical to CaM kinases II and IV. Homology is also high in the regulatory domain which consists of the autoinhibitory and calmodulin binding regions. As such, we are able to predict the amino acids that comprise the autoinhibitory region of I43 and should be able to easily generate a constitutively active truncated mutant which will aid in determining kinase function. Structural analysis also reveals the presence of two unique regions of I43 at both the extreme N- and C-terminus.

Structural analysis of A32 confirms the presence of a serine/threonine kinase domain, but no other conserved protein motifs have been found using this method of analysis. This may be due to the fact that A32 is unique and does not contain conserved motifs, or possibly because the conserved motifs of A32 are not detectable by homology searches. As newer programs become available for this type of homology analysis, conserved motifs may be found within A32. Further functional analysis of A32, however, is not dependent upon analysis of amino acid structure and this portion of the project will be ongoing.

Technical Objective II: Characterize temporal and spatial expression patterns of novel kinases.**Task 1: months 6-24: Characterize temporal expression patterns.**

In order to determine if the expression of the kinases is regulated during mammary gland development, RNA was isolated from mammary glands of FVB mice at various developmental timepoints. RNase protection analysis was used to determine the relative expression levels of A32 and I43 at each of the timepoints. As shown in figure 1, I43 levels rise during pregnancy and peak late in pregnancy when epithelial cells are differentiated. Conversely, A32 levels are highest early in pregnancy as epithelial cells are differentiating and then drop later in pregnancy.

A.



B.

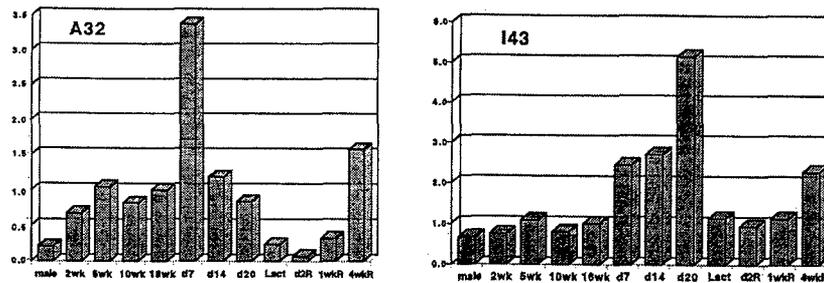


Figure 1: Expression of A32 and I43 during mammary gland development. A) RNase protection B) expression levels normalized to actin.

In order to determine the effect of ovarian hormones on A32 expression levels, ovaries were removed from adult female mice, estrogen and progesterone were injected sub-cutaneously for two weeks, and mammary glands were harvested. As shown in figure 2, I43 levels were not affected by hormone injection. However, A32 levels were dramatically increased by the presence of both progesterone and estrogen, but not by either hormone alone. These results are consistent with A32 playing a role early in pregnancy and I43 later during pregnancy.

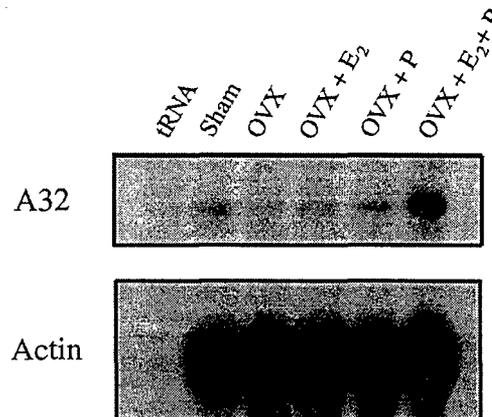


Figure 2: A32 expression in mammary glands of ovariectomized (OVX) mice treated with estradiol (E₂), progesterone (P), or both.

In order to investigate the expression of A32 and I43 in mammary epithelial cell lines, RNA was isolated from a panel of normal and tumor cell lines arising in transgenic animals. Neither kinase was detected in the fibroblast cell line, 3T3, suggesting that mammary gland expression is epithelial-specific (figure 3). Expression of I43 was detected only in tumor cell lines arising in animals overexpressing either the *c-myc* or *int2* oncogenes. A32, however, was not detected in this sub-set of cell lines, yet was expressed in every tumor cell line derived from transgenic animals overexpressing *neu* or *Ha-ras* oncogenes. These results suggest that A32 and I43 may be markers for different subsets of mammary epithelial cell subtypes that may be preferentially transformed by the respective oncogenes.

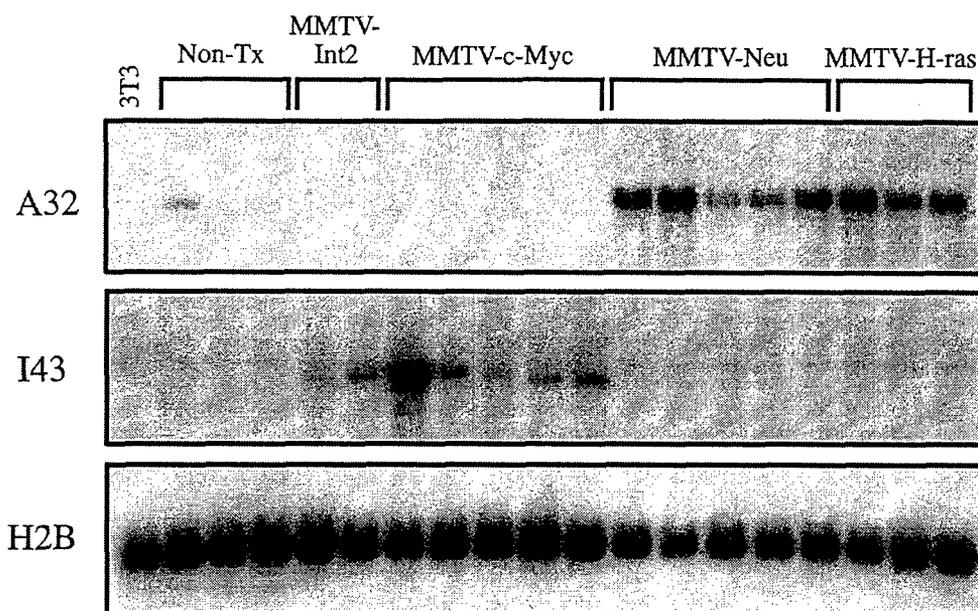


Figure 3: Expression of A32 and I43 in normal cell lines and cell lines derived from tumors arising in transgenic mice overexpressing various oncogenes.

Task 2: months 1-24: Characterize the spatial expression pattern of the novel kinases.

In order to determine the spatial expression pattern of the kinases in the mammary gland, frozen OCT blocks were sectioned and hybridized to 35S-labeled sense and antisense riboprobes as described (Marquis, et al.). *In situ* hybridization confirms the up-regulation of A32 early in pregnancy and I43 later in pregnancy (data not shown). Interestingly, both kinases appear to be expressed in a subset of epithelial cells since silver grains are not uniform over the entire epithelium, rather appear as punctate spots over a fraction of epithelial cells.

Technical Objective III: Determine the functional roles of the novel kinases.

Task 1: months 12-24: Define the role of the novel kinases in differentiation.

Both *in vivo* and *in vitro* differentiation systems are being utilized to determine if overexpression of A32 or I43 affects normal differentiation pathways. PC-12 cells treated with NGF undergo differentiation characterized by the appearance of neurite outgrowth. During this differentiation process, A32 levels drop while I43 levels rise (data not shown). Both genes under the control of the CMV promoter have been transfected into PC-6.3 cells, a subset of PC-12 cells, in order to determine if overexpression of either gene affects normal NGF-induced differentiation. Currently, we are in the process of selecting stable clones from this transfection. Mention HC-11 cells??

In order to determine if overexpression of A32 or I43 affects normal mammary gland development, we have generated transgenic mice with each gene under the control of the mouse mammary tumor virus (MMTV) promoter which is commonly used to give mammary gland specific expression. We are currently analyzing transgene carriers (5 animals for A32 and 6

animals for I43) to determine the number of founder animals for each kinases. Founder animals will be bred and analyzed to determine if mammary-specific transgene overexpression affects normal mammary gland development.

Task 2: months 12-48: Define the role of the novel kinases in proliferation.

In order to determine if A32 or I43 affects proliferation, we have transfected each gene, under the control of the CMV promoter, into a normal fibroblast cell line (3T3) and a normal mammary epithelial cell line (NMuMG). Stable clones are under selection and will be compared to vector-only control clones with respect to BrdU and 3H-thymidine incorporation to determine the effect on cell growth rates. Since I43 levels rise 3-6 fold in confluent cells (data not shown), we might expect overexpression of I43 to decrease growth rates. However, this effect has not been observed thus far in the experiment.

In our transgenic animals, we will also be looking for gross morphological changes that may result from decreased or increased proliferation rates due to overexpression of our transgene. This type of analysis, however, may not be able to distinguish between proliferation effects and differentiation effects. Therefore, a tissue culture system may be necessary to differentiate between the two.

Task 3: months 18-48: Determine the role of the novel kinases in carcinogenesis.

In order to determine if 3T3 cells form foci when transfected with either of the novel kinases, cells were transiently transfected with full-length clones under the control of the CMV promoter and refed every other day for two weeks. Neither A32, nor I43 was able to form foci in 3T3 or NMuMG cells under these conditions. To determine if overexpression allows cells to grow in soft agar, the transient transfection will be repeated and cells will be assessed for their ability to grow in an adherence-independent fashion.

Transgenic animals which overexpress A32 and I43 in a mammary-specific manner will be monitored for the presence of mammary tumors. Since the latency of tumor formation may be very long, this experiment will be ongoing indefinitely.

As shown above, I43 and A32 are expressed in tumor cell lines in an oncogene-specific manner (figure 3). In order to determine if this expression pattern is a direct effect of transgene expression (A32 and I43 are down-stream) versus transformation of an epithelial cell-type, we are investigating the expression of each of the novel kinases in non-transformed mammary epithelium from MMTV-c-myc and MMTV-c-neu animals. We are in the process of harvesting mammary glands from animals at various developmental timepoints in order to determine if normal expression of A32 and/or I43 is altered as a result of transgene expression. In addition, we are harvesting primary tumors from these transgenic animals in order to determine if A32 and I43 are up-regulated in the tumors arising in MMTV-c-neu and MMTV-c-myc animals respectively. These experiments should tell us if A32 and I43 are down-stream of the oncogenes, or if they seem to be markers of epithelial sub-types which are differentially transformed by the oncogenes.

CONCLUSIONS

In the first year of this project, we have completed the majority of our first two technical objectives. Full-length clones for both A32 and I43 have been isolated and homology to known kinases has been determined. The 1.5kb cDNA for I43 codes for a protein of 343 amino acids that is highly homologous to the calcium-calmodulin (CaM) family of serine/threonine kinases. Although the homology is high, I43 is novel and may even represent a distinct class within the CaM kinase family. A full-length clone for A32 has been significantly more difficult to obtain due to the large size. Recently, however, multiple cDNA clones have been isolated that are similar in size to each other and are approximately the correct size as determined by Northern analysis. In addition, comparison of mouse and human clones reveals high homology in the putative coding region with much lower homology in the putative UTRs. The full-length clone for A32 codes for a protein of 714 amino acids, contains a 5' UTR of at least 60bp and a 3kb 3' UTR. A32 is homologous to the SNF1 family of serine/threonine kinases both in the kinase domain and in a short region C-terminal to the kinase domain known as the SNF1 homology domain. Outside of these known regions, A32 appears to be novel and shares no homology to other known proteins.

We have determined the temporal and spatial expression patterns of these kinases by Northern analysis, RNase protection assays, and *in situ* hybridization. Expression analysis of I43 suggests a role late in pregnancy possibly during differentiation since expression levels peak late in pregnancy and are also induced during NGF-treatment of PC-12 cells. Conversely, A32 levels peak early in pregnancy and decrease when PC-12 cells are treated with NGF suggesting a role early in differentiation. Expression studies such as these will be important in helping to determine the biological function of these molecules *in vivo*.

In order to determine the functional role of these kinases, we have begun to overexpress full-length clones in a variety of systems. Several stable cell-lines are being selected in order to determine changes in both differentiation and proliferation in *in vitro* tissue culture systems. In addition, transgenic mice have been generated which overexpress the kinase of interest in a mammary-specific fashion. These transgenic animals will be used to study the effects of kinase overexpression *in vivo*. Differentiation, proliferation, and effects on carcinogenesis susceptibility will be determined throughout the remainder of this project. Over the period of the next few years, all of the objectives originally outlined in our proposal should be met in a timely fashion. As such, we believe these studies will contribute to our understanding of the role played by I43 and A32 in mammary gland development and carcinogenesis.

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21 JUN 2001

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PHYLLIS M. RINEHART
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