The Signal Peptide of Mouse Mammary Tumor Virus-Env: A Phosphoprotein

Tumor Modulator

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Running Title: The signal peptide of MMTV-Env: a tumor modulator

Key words: Signal Peptide, MMTV, Nucleolus, Nucleophosmin (B23), L5.
Abstract

Mouse mammary tumor virus (MMTV) is associated primarily with mammary carcinomas and lymphomas. The signal peptide of the MMTV-envelope precursor is uniquely targeted to nucleoli of cells that harbor the virus, where it can function as a nuclear export factor for intron-containing transcripts. Antibodies to this signal peptide, which we refer to as p14, were previously shown to label nucleoli in a subset of human breast cancers. To look for additional cellular functions of p14, different mutants were ectopically expressed in the MCF-7 human breast cancer cell line. This approach identified motifs responsible for its nucleolar targeting, nucleocytoplasmic shuttling, target protein (B23, Nucleophosmin) binding, and phosphorylation at serine 18 and 65 both in situ and in vitro. To test the role of these phosphorylation sites, we performed in vivo tumorigenesis studies in SCID mice. The findings demonstrate that the p14-Ser65Ala mutation is associated with impaired tumorigenicity while the p14Ser18Ala mutation is associated with enhanced tumorigenicity. Microarray analysis suggests that phosphorylation at serine 18 or at serine 65 is associated with transcriptional regulation of the L5 nucleolar ribosomal protein (a p14 target) and the Erb-B signal transduction pathway. Taken together, these results show that the phosphorylation status of p14 determines whether it functions as a pro-oncogenic or anti-oncogenic modulator.
Introduction

Mouse mammary tumor virus (MMTV) is a type B retrovirus associated primarily with mammary carcinomas and lymphomas in laboratory mice (1, 2). During the 1970s, MMTV was suggested to play a role in the etiology of human breast cancer when claims were first made that MMTV antigens were involved in this disease (3). A new wave of interest in this issue started in the mid-1990s when MMTV DNA sequences were first identified, using PCR technology, in 38% of human breast cancer samples, but not in healthy tissue (4). Despite the lack of incontrovertible evidence for a causal relationship between MMTV and breast cancer, and doubts whether MMTV is a tumor virus or just a “rumor” virus in the context of this disease [(5) and references therein], a number of reports from different groups do suggest an association between MMTV (or HMTV – Human Mammary Tumor Virus) sequences and breast cancer in up to 74% of women with the disease (related to geographical distribution) (6-8). Indeed, it has recently been reported (9) that inflammatory breast cancers from American women show a higher incidence of HMTV-related viral sequences (71%) than sporadic breast cancers, and that the presence of MMTV-env-like exogenous sequences is strictly related to tumor progression (10). Additional support for possible involvement of the virus in breast cancer comes from reports claiming that: MMTV can infect and spread through a culture of human mammary cells (11, 12); the MMTV env protein, through a specific sequence motif, can initiate changes that are consistent with transformation of mammary epithelial cells (13); cells isolated from ascites or pleural effusions of patients with metastatic breast cancer contained MMTV sequences in their DNA, expressed the MMTV Env protein, and showed β-retroviral particles by electron
microscopy, similar to the mouse virus (14). The same group recently reported
detection of HMTV proteins in human breast cancer cells (15).

Previously, we demonstrated that the signal peptide (98 N-terminal amino acids) of the Env-precursor protein of MMTV is localized in nucleoli of murine T-cell lymphomas that harbor the virus (16, 17). This peptide was initially named MMTV-p14 (p14, for short) due to its electrophoretic mobility. Its mass was subsequently determined by mass spectrometry to be 11 kDa (17). The nucleolar localization of p14 is not limited to murine lymphomas, as it also occurs in mouse mammary carcinoma cell lines that harbor MMTV (18). Cell lines devoid of MMTV (either lymphoma or mammary carcinoma) do not show any cross-reaction with anti-p14 antibodies. Furthermore, using antibodies generated against recombinant p14, this protein was identified in nucleoli of a sub-set of human breast cancers (18). Comparing the sequence of p14 with the parallel sequence of a provirus isolated from human breast cancer, revealed 86% homology between the two (18).

N-terminal signal sequences are known to mediate targeting and insertion of nascent secretory and membrane proteins into the endoplasmic reticulum (ER). Following targeting of the 74 kDa envelope protein precursor of MMTV to the ER, the signal sequence is cleaved, and the envelope protein is further glycosylated and processed to give the viral gp52 and gp36 envelope glycoproteins. No additional functions were previously proposed for MMTV-p14, as it was assumed to be degraded. Subsequent to our initial reports, it was shown that p14 is also the signal sequence of a splice variant of MMTV-env (termed REM) functioning as a nuclear export factor for intron containing transcripts (analogous to the role of HIV-Rev protein) (19, 20). A recent study suggested a novel pathway, whereby this signal sequence can be released from the ER, accumulate in the nucleoli and thus, fulfill a
post-targeting function in a different compartment (21). Although a variety of viral proteins, such as HIV-Rev, HTLV-Rex and others, enter the nucleus and accumulate in the nucleoli of infected cells, our studies demonstrated, for the first time, that the signal peptide of a retrovirus envelope protein survives to accumulate in the nucleolus. This was also the first time that any MMTV protein was observed in this compartment. Following these studies, nine p14-binding proteins have been identified (18). Four of these: B23 (Nucleophosmin), L5, La and Acidic ribosomal binding protein are nucleolar proteins involved in a variety of cellular processes. Interestingly, p14 is not an isolated case of a viral signal peptide translocating to the nucleolus: it was recently reported that the human endogenous retrovirus HERV-K (HML-2) encodes a stable signal peptide, analogous to p14, which also localizes in nucleoli (22). Collectively, these, and our previous findings, support the concept of signal peptides as retroviral modulators of their host cell physiology.

Materials and Methods

Cell culture

All materials were from Invitrogen (Carlabad, CA), unless otherwise noted. MCF-7 human breast cancer cells (a kind gift of Dr. Nissim Benvenisty) were grown in DMEM, with 10% fetal calf serum, 1% L-Glutamine, 50 mg/L streptomycin and 50,000 units/L penicillin. Cells were grown in culture flasks (Thermo Fisher Scientific, Rochester, NY) at 37°C in 5% CO₂. The original aliquot was propagated and frozen in aliquots. All experiments were performed from these aliquots, which where not grown for over three months. Recombinant cell lines, after selection, were treated the same way.
N-Terminal His-Tagged p14
cDNA clone 66b (16) was used as a template and the PCR fragment corresponding to
the p14 sequence was ligated into pET-22b(+), parallel2 (Novagen, Madison, WI).
The resulting plasmid was confirmed by sequencing and the expressed His-tagged
p14 protein was purified on a TALON (Clontech, Palo Alto, CA) affinity column and
purity confirmed as previously demonstrated (18).

Eukaryotic expression of p14
cDNA clone 66b was also used as a basis for eukaryotic expression. The sequence
was ligated into the pTRE2hyg plasmid, validated, and transfected into MCF-7 cells
containing the pTet-off plasmid (Tet-off Gene Expression System - Clontech,
Mountain View, CA). In this system, addition of doxycycline, a tetracycline
derivative, turns off expression of the plasmid. Expression of p14 was confirmed and
the cells cloned. Mutations (see figure 1A) were created with the primers listed in
Supplemental data, Tables 1 & 2, using the QuikChange II site directed mutagenesis
kit from Agilent Technologies (Santa Clara, CA).

Western Blots
Cells were lysed by SDS-PAGE sample buffer, separated by 15% SDS PAGE and
transferred to nitrocellulose. p14 (and p21) were visualized using the polyclonal anti-
p14 antibody (18), HRP–linked donkey anti-rabbit antibody (Jackson Laboratory, Bar
Harbor, ME) and Pierce Super Signal (Thermo Fisher Scientific, Rockford, IL).
Immunoprecipitation and Immunofluorescence

These were carried out as previously described (19, 20).

In Vitro Phosphorylation Assays

PKC – 10 μg of recombinant p14 (see above) or of Histone (Sigma, St. Louis, MO) was incubated with 2.5 ng of PKC (EMD Chemicals, Gibbstown, NJ) in the presence of 1.25mM EGTA, 50mM MES (PH=6), 12.5mM MgCl₂, and 1 mM ATP at 30°C for 1 hr. Phosphorylation was determined by Western blot using α-phosphoserine antibodies (Abcam, Inc, Cambridge, MA).

CK2 - 10 μg of recombinant p14 was incubated with 500 U of CK2 (New England Biolabs, Ipswich, MA) using buffer supplied with addition of 1 mM ATP at 30°C for 1 hr. Phosphorylation was determined by Western blot as described above.

Fluorescence recovery after photobleaching (FRAP)

MCF-7 cells expressing GFP-p14 were grown in standard conditions. Individual nucleoli were photobleached and the fluorescent recovery of p14 in the nucleolus was followed using a FV-1000 confocal microscope (Olympus, Japan).

Heterokaryon assay

This was performed as described (23). MCF-7 and NIH-3T3 cells were plated together. The medium was replaced with 300 μl medium containing 100 μg/ml cycloheximide. After 30 min, 300 μl of 50% PEG3350 (Sigma, St. Louis, MO) was added for 105 sec at room temperature. Wells were washed 3X with PBS, and growth medium containing 100 μg/ml cycloheximide was added. Cells were incubated at 37°C, 5% CO₂ for four hrs and analyzed by immunofluorescence as described above, except that nuclei were stained with Hoechst (Sigma, St. Louis, MO).
leptomycin B (30ng/ml) (Invitrogen, Carlsbad, CA) was added, the incubation times were 2.5 hrs before PEG addition and 5 hrs after.

MCF-7 cell injection into SCID mice

MCF-7 cells (naïve or ectopically expressing p14) were washed and resuspended in PBS at 2 X 10^6 cells/100μl. 50 μl was injected bilaterally into each of the third mammary fat pads of female SCID Balb/c mice 6-8 weeks old (Harlan, Israel). Mice were held in an SPF facility (AAALAC accreditation #1285), treated and euthanized following ulceration of the tumors in accordance with NIH guidelines.

Microarray analysis

Total RNA isolation was performed with the perfect pure RNA cell & tissue kit (5PRIME, Hamburg, Germany). Purity and concentration were determined by absorbance and by RNA gel electrophoresis. For hybridization to the GeneChip Human Gene 1.0 ST Array (28,869 genes) (Affymetrix, Santa Clara, CA), two independent RNA preparations were made from each of the tested MCF-7 lines.

Microarray raw data are in the Supplemental files.

Statistical analysis of microarrays was preformed with assistance of The Genomic Data Analysis Unit of Hadassah Medical School, Hebrew University of Jerusalem. The normalized mean of the mRNA sample repeats was compared to the control cells mean using a threshold of ≥ 2-fold and P-value of ≤ 0.05. The resultant gene lists were subjected to cross analysis and visualized using a Volcano diagram (Partec Genomics Suite, Partec Inc, St. Louis MO). These genes were further analyzed using the Ontoexpress pathway program (http://vortex.cs.wayne.edu) and individual gene
examination, in order to identify genes or pathways that were significantly affected by
the presence of p14.

Quantitative real-time PCR
Quantitative real-time PCR intron spanning primers were designed, using Primer
Express 2.0, for the selected genes, RPL5 (F: 5’-TAT GCA CAC GAA CTG CCA
AA-3’ R:5’-CCA-TGC CAA ACC TAT TGA GAA G-3’), ErbB4 (F:5’-GGC-AAG-
ATA-TTG-TTC-GGA-ACC-C-3’ R:AGC-AAC-GGC-CAG-TAC-AGG-ACT-T-3’),
and the housekeeping gene GAPDH (F:5’-TGC-ACC-ACC-AAC-TGC-TTA-GC-3’
R:5’-GGC-ATG-GAC-TGT-CGT-CAT-GAG-3’), with expected product sizes of 100
to 130 bp. RNA was then treated with DNAse, and reverse transcribed with
SuperScript First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA).
Amplification was conducted using KAPA SYBR FAST (Kapa Biosystems, Woburn,
MA), according to the standard mix protocol. The samples were run using ABI prism
7900HT machine (Applied Biosystem, Foster City, CA.). Protocol consisted of 2 min,
50°C reverse transcriptase followed by 10 min at 95°C. 40 cycles of 15 sec at 95°C,
and 1 min at 60°C) were performed. A melting curve was used to confirm the
specificity of the PCR products. Experiments were performed in triplicates.
PCR reaction efficiency was calculated for each primer pair with five dilution points
of the calibrator sample to validate primers. Fold change in expression of each gene
was calculated using the ΔΔCt method. ΔΔCt = [Target gene in p14 sample -
housekeeping gene in the same sample] - [ Target gene in control - housekeeping gene
in control]. Fold Change= 2^−ΔΔCt (24). Significance of the data was confirmed using
Results

In view of the recent findings on MMTV involvement in breast cancer, and the immune reactivity of some breast cancer tissue arrays towards anti-p14 antibodies (6, 18), we examined the role of p14 in the biology of breast cancer cells devoid of MMTV. Inducibly expressed p14 was transfected into MCF-7 cells as described in Materials and Methods. Immunofluorescence and western blot analysis of the p14-expressing cell line demonstrated that p14 was expressed and localized in the nucleolus (Fig. 1 C and F), similar to its localization in murine lymphomas and mammary carcinomas that harbor MMTV [Fig. 1E and F and (18)]. As expected, incubation of this cell line with Doxycycline (Dox) reduced p14 expression to very low levels (Fig. 1D and F). Nevertheless, the obvious leakiness of the system prevented us from using Dox-treated cells as internal negative controls.

Intracellular localization of mutant p14’s.

The p14 amino acid sequence was analyzed using the Prosite (www.expasy.org/tools/scanprosite) and MyHits (http://myhits.isb-sib.ch/cgi-bin/motifsscan) sites to determine putative functional motifs (17). We then created MCF-7 cell lines stably expressing a battery of mutated p14 sequences that include point mutations at putative post-translational modification and nuclear localization sites (NLS), and deletions along the sequence of p14, including the NLS region (Fig. 1A). These were double-stained for p14 and for B23, a nucleolar shuttling protein that also binds p14 (17, 18). As can be seen in Figure 2, wild-type p14 localized into the nucleolus, co-localizing with B23. The mutations in the region of the NLS, however, showed a gradient of differing localization profiles (Fig. 2): The double point mutation HHNLS, in which histidines replaced two consecutive basic amino acids
(R$_{32}$R$_{33}$) in the first half of the bipartite NLS, showed some re-localization out of the
nucleolus and into the nucleus. The double point mutation EDNLS, in which
Glutamate and Aspartate replaced two consecutive basic amino acids (K$_{31}$R$_{32}$), was
localized to the nucleus to a larger extent. Deletion of the first half of the NLS (R$_{29}$ –
R$_{34}$) moved almost all of the p14 out of the nucleolus, leaving the majority in the
nucleus, with a significant amount of the mutant p14 remaining in the cytoplasm.
Deletion of the entire NLS (R$_{29}$ – R$_{45}$) resulted in all of the p14 remaining in the
cytoplasm. This confirms that the sequence previously identified as the NLS is
responsible for localization of p14, not only to the nucleus, but also to the nucleolus.
Mutations outside of the NLS (including the putative CK2 and PKC phosphorylation
sites) did not affect p14 localization to the nucleolus (not shown). We also found that
stop codon mutations (Fig.1A) did not express well enough to warrant further
experiments.

**Binding of p14 to B23.**

The different p14 mutants were further tested as to their binding capacity to B23. Fig.
3A shows that B23 co-immunoprecipitates with p14-wt, as well as with the PKC$^-$
mutant (and all other deletion mutants – not shown), but not with CK2$^-$ and del1
mutants. In order to confirm this result, B23 was immunoprecipitated and the
resultant immunoprecipitate western blotted vs $\alpha$-p14 antibodies (Fig. 3B). In this
experiment, too, CK2$^-$ and del1 mutations showed no interaction with B23. These
findings suggest that Serine$_{65}$ in the putative CK2 phosphorylation site is involved in
the interaction with B23. The del1 sequence also participates in the interaction with
B23. However, since the mutation S18A in the putative PKC phosphorylation site
(included in the del1 sequence, see Fig.1A) is not involved in B23 binding, it is likely
that a shorter sequence of del1 participates in this interaction.
Dynamics and nuclear export of p14.

To determine the mobility of p14 within the nucleus, MCF-7 cells were stably transfected with a GFP-p14 expressing plasmid and subjected to Fluorescence Recovery after Photobleaching (FRAP). The mobility of p14 was high, and recovery was essentially complete within a minute (Fig. 4A). A similar observation (recovery within a minute) was made upon transfection of Hela cells with GFP-p14 and with Rev-GR-GFP in a well described nuclear import export assay as a control (not shown).

To study the nuclear export of p14, heterokaryon analysis was applied. To that effect, p14-expressing MCF-7 cells were fused with NIH-3T3 cells (murine origin with no MMTV background). The nuclei of these two cell types can be visually distinguished (23), so that the export of p14 from human to murine nuclei in the heterokaryon can be monitored. As can be seen in figure 4B, the export of p14 from MCF-7 nuclei to NIH-3T3 nuclei in the fused cells was identifiable whether the MCF-7 cells were expressing p14-wt, CK-2–, or del1 mutations. Note also that the nuclei of the mouse-mouse heterokaryon in the CK2 panel are devoid of p14. Identical results were seen with MCF-7 cells expressing PKC– and del2 mutations (not shown). As B23 does not bind to the CK2– and del1 mutant p14’s (see Fig.3), these findings suggest that B23 is not involved in the nuclear export of p14. Leptomycin B, an inhibitor of nuclear export, prevented the accumulation of p14 in the murine nuclei of the heterokaryons (Fig. 4B), indicating that p14 is actively exported from the nucleus via a CRM1-dependent mechanism [see also (19)].

p14 is a phosphoprotein

To determine whether the putative CK2 and PKC sites of p14 are endogenously phosphorylated, ectopically expressed p14 wild type, and the point
mutations Ser18Ala (PKC−) and Ser65Ala (CK2−) were immunoprecipitated from MCF-7 cell extracts with α-p14 antibodies. The phosphorylation level of these proteins was determined by Western blot using α-phosphoserine antibodies. As can be seen in Fig. 5A, while both wild type and the two mutant proteins were phosphorylated, the wild type p14 was more highly phosphorylated than either mutant alone. A double mutant (Ser18Ala, Ser65Ala), containing point mutations in both serines was not phosphorylated endogenously (Fig. 5A). These findings are consistent with both serines being endogenously phosphorylated.

We next attempted to phosphorylate the PKC− and CK2− mutants in vitro, using purified CK2 and PKC enzymes (see Materials and Methods). As can be seen in Fig. 5B, CK2 could phosphorylate the purified p14. PKC does not seem to phosphorylate p14 in vitro, suggesting that another serine-kinase may be involved in phosphorylation of this site. Taken together, these findings are consistent with p14 being phosphorylated by different kinases at serines 18 and 65.

Additionally, preliminary results in our laboratory have indicated that the ribosomal protein L5 is differentially immunoprecipitated with the various phosphorylation mutants of p14.

The phosphorylation state of p14 alters tumorigenicity. The phosphorylation mutants (PKC−, CK2−, and double mutant PKC−-CK2−), as well as wt-p14 expressing, and naïve MCF-7 cells, were injected (1 X 10⁶ cells) into each of two mammary fat pads of female SCID mice and followed thereafter for development of progressive tumors (see Materials and Methods). This inoculum of cells was chosen because it allows us to analyze whether mutant p14’s either impair or enhance the tumorigenicity of affected cells relative to naïve MCF-7 cells.
MCF-7 cells expressing the p14-PKC\(^{-}\) construct developed progressive tumors in all
mice (n=5) (Fig.5C). In one of these mice, tumor cells infiltrated the anterior
chambers of both eyes, thus pointing towards enhanced metastatic potential of MCF-7
cells expressing p14 with the PKC\(^{-}\) mutation. On the other hand, mice injected with
either CK2\(^{-}\) or double CK2\(^{-}\)-PKC\(^{-}\) mutants did not develop any tumors for at least
150 days, when the experiment was terminated. Both naïve MCF-7 and p14 wt cells
developed progressive tumors in 3/5 mice. The time course of tumor appearance in
the p14 wt injected group was delayed relative to the naïve MCF-7 cells. However,
two mice from the p14 wt injected group developed solid tumors distant from the sites
of inoculation

A chi-square test indicates that the proportions of tumor positive mice are not the
same among the five different cell types (\(\chi^2 = 15.26, P = 0.0025\)). Due to the
relatively small sample size (N=5), the \(P\) value was estimated by computer
simulations – \(10^4\) random samples were drawn under the null assumption of
independence between treatments and tumor positive mice, and the proportion of
samples with a chi-square value larger than, or equal to the observed chi-square
statistic, was taken as an estimate of the real \(P\) value

More specifically, the proportion of tumor positive mice injected with p14 PKC\(^{-}\) cells
is significantly larger than the proportions for those injected with p14 CK2\(^{-}\) and
double mutant cells (Fisher's Exact Test, \(P = 0.004\)), whereas the proportions for mice
injected with naïve and p14 wt cells have an intermediate value (though not
significantly different from the two extremes).

The above findings are consistent with the notion that the phosphorylation status of
p14 is instrumental for regulating the tumorigenic potential (both positively and
negatively) of MCF-7 cells that express this protein. We note that CK2 over-
expression has been linked to increased tumorigenicity in murine cancer (10).

Transcriptional regulation by p14.

The above findings linking phosphorylation of p14 to tumorigenic potential prompted
us to further examine the molecular basis of these changes. L5 was previously
identified by us as a target protein for p14 (18). It also binds MDM2 in response to
nucleolar stress (25), and another p14 target, B23, which is essential for the nuclear
export of L5 (23). We attempted to determine if L5 would be differentially expressed
by cells expressing either p14 wt or the various phosphorylation mutants. Real time
PCR (Fig. 6D) showed a two-fold increase in L5 RNA in MCF-7 cells expressing p14-
wt (relative to naïve MCF-7 cells), while, in MCF-7 cells expressing p14-PKC−, L5
showed a 6.7 fold increase in transcription compared to naive MCF-7 cells. CK2−
cells showed a 1.5 fold decrease (relative to naïve MCF-7 cells) in L5 RNA. Thus the
ratio of L5 expression in PKC− cells to CK2− is approximately 10 fold.

To test whether expression of p14 and its phosphorylation variants play a more
extensive role in transcriptional regulation, RNA was extracted from independent
duplicates of MCF-7 cells stably expressing the WT, PKC− or CK2− mutant p14’s and
subjected to microarray analysis (see Materials and Methods). As the PKC− and the
CK2− cells demonstrate the opposite extremes of tumorigenicity (see Figure 5C), the
transcriptional profile of these two cell lines were compared. Volcano analysis of the
data (Fig. 6A) demonstrates that 142 genes (see Table 3 - supplemental data) are
transcriptionally regulated (up, down) by the PKC− vs CK2− mutant p14s. The 18 most
statistically significant genes, along with their cellular functions, are listed in Table 1.
Figures 6 B & C are a graphical analysis of the absolute expression of these genes in
the four cell types. As can be seen, the differences in the expression profile are not
random, as the changes in gene expression are mostly specific to a particular cell line. Thus, most of the genes with a higher expression profile in CK2\(^\prime\) (or PKC\(^\prime\)) cells are higher only in CK2\(^\prime\) (or PKC\(^\prime\)) cells.

The epidermal growth factor receptor, ErbB4, was chosen for quantitative confirmation of the chip analysis. This gene was found to change significantly (decrease over 10 fold in the microarray analysis) only in the CK2\(^{-}\) mutant cells (Fig 6C). Additionally, many of the components of the ErbB4 signaling pathway - 6 out of 14, as well as 11 out of 87 components of the more intricate ErbB pathway, were found to be differentially expressed in the various transfectants (see Supplemental Data). Furthermore, it was found that an integration site in cells infected with MMTV was in an intron of ErbB4 (Stanislav Indik and Walter H. Gunzburg - Personal Communication). These pathways are instrumental in growth control of mammalian cells, as well as in mammary carcinogenesis and breast cancer. Real time PCR (Fig. 6D) showed a small (1.3 fold) increase in ErbB4 RNA in MCF-7 cells expressing p14-wt compared to naive MCF-7 cells. The PKC\(^{-}\) cells showed a 3.3 fold increase in ErbB4 RNA. The CK2\(^{-}\) cell line expressed a 6.4 fold decrease of ErbB4 RNA. The difference between the least tumorigenic (CK2\(^{-}\)) and the most tumorigenic (PKC\(^{-}\)) cells with regards to ErbB4 RNA is, therefore, 21 fold. The quantitative data suggest a correlation between the transcriptional activation of ErbB4, as well as L5, with the enhanced tumorigenicity of p14-PKC\(^{-}\) expressing cells. A correlation is also evident between the transcriptional inhibition of ErbB4 and L5, with the impaired tumorigenicity of the p14-CK2\(^{-}\) expressing cells. Note that L5 is not only transcriptionally regulated by p14, but is also a binding partner of p14 (18).

Thus, the phosphoprotein p14, when ectopically expressed in MCF-7 cells lacking MMTV, modulates tumorigenicity. Phosphorylation of p14 appears to be a
critical regulator of this process. Mutants lacking the CK2 phosphorylation site show diminished tumorigenic potential. This mutation is also associated with transcriptional deregulation of the ErbB signal transduction pathway and changes in the expression of nucleolar/ribosomal genes. These findings suggest that p14 acts independently of other MMTV viral products to induce changes in cellular physiology.

Discussion

The signal peptide of the Env-precursor protein of MMTV, p14, is localized to the nucleoli of cells that contain the whole virus or transgenically express p14. Furthermore, p14 is a highly mobile phosphoprotein within the nucleus, and its nuclear export is CRM1 dependent and independent of its nucleolar target protein B23. The phosphorylation status of Serines 18 and 65 play critical roles in modulating the tumorigenic potential of MCF-7 cells that express p14. Expression of p14 alters transcription of a number of genes, including the ribosomal protein L5 (a formerly defined target of p14), and the ErbB4 pathway involved in breast cell growth and differentiation. The expression levels of these proteins correlate with the tumorigenicity of MCF-7 cells that express mutant p14.

In recent years, it has been established that in addition to its role in rRNA synthesis and ribosome biogenesis, the nucleolus is involved in a variety of cellular functions, including regulation of the cell cycle, senescence, gene silencing, and sensing of cellular stress (26-31), as well as in viral replication (32, 33). The relationship between the nucleolus, ribosome biogenesis, and cancer has been the topic of a recent review (34) where some evidence to that effect has been documented. Still, the authors caution that most of the proteins involved in ribosome
biogenesis (B23 for example) have extra-ribosomal functions that may support
tumorigenesis independent of ribosome function and mRNA translation.

Although a variety of viral proteins are translocated into the nucleolus
following viral infection (33), we were the first to demonstrate that the signal peptide
of MMTV-Env precursor is also localized to this compartment(16, 17). It turns out
that this is not an isolated case, as it was recently demonstrated that the leader
sequence of HERV-K is also localized in the nucleolus (22). Thus, it cannot be
excluded that other viral signal sequences may be targeted to the nucleolus.

Previously, the nucleolar proteins B23 (Nucleophosmin, numatrin, NO38) and
L5 have emerged as proteins interacting with p14 (18). Here, we extended these
findings by defining those p14 domains involved in B23 binding. One of these is
Ser65 in the CK2 phosphorylation site. Thus, it is likely that phosphorylation of this
Serine by CK2 enhances B23 binding to subsequently regulate B23-related functions.

B23 is a multifunctional nucleolar phosphoprotein that shuttles continuously between
the cytoplasm and nucleolus. It is essential for ribosome biogenesis and is thought to
act as both a protooncogene and a tumor suppressor (35, 36). Moreover, B23
maintains genomic integrity by controlling DNA repair mechanisms and centrosome
duplication during mitosis. It has also emerged as a regulator of the
p53/p14ARF/MDM2 pathway, stabilizing p53, thus playing a role in mediating the
cellular stress response (35, 37-39). B23 has been strongly implicated in
hematopoietic malignancies, where translocations and mutations involving the gene
have been reported in various lymphomas and leukemias, giving rise to fusion
proteins or mutant products (40).

For ribosomal protein L5, qRT-PCR findings provide additional support to the
previously documented interaction between p14 and this nucleolar protein (18). Here,
the level of L5 mRNA directly correlates with the tumorigenicity of MCF-7 cells transfected with different p14 mutations, consistent with transcriptional regulation of L5 by p14. The ribosomal L5 protein is a 60S subunit protein that chaperones the 5S rRNA into the nucleolus and out into the cytosol (41). Interestingly, B23 has been shown to directly interact with L5 and play an essential role in its nuclear export (23). Interaction of these two proteins in a more general context was reported recently, when B23 was implicated in directing the nuclear export of both 40S and 60S ribosomal subunit proteins (42). L5 has also been reported to inhibit MDM2-mediated p53 ubiquitination and degradation, by inhibiting the E3 ubiquitin ligase activity of MDM2 (25, 43, 44).

Since B23 and L5 both play a role in the regulation of a major stress response pathway, and since both molecules interact with MMTV-p14, we propose that, through its interaction with these two nucleolar proteins, MMTV-p14 (and therefore MMTV) can affect the cellular stress response and its outcome (including apoptosis, cell cycle regulation, tumorigenesis etc.), thus adding an additional level of complexity to the regulation of these interactions in the nucleus. How the phosphorylation status of p14 is involved in these interactions is presently unknown.

In addition to L5, ErbB4 is another gene whose transcriptional regulation by p14 is phosphorylation dependent. The upregulation of ErbB4 transcripts correlates with the tumorigenic potential of MCF-7 cells expressing different p14 mutants. ErbB4 is a member of the epidermal growth factor receptor family that mediates cell proliferation and differentiation and mammary gland differentiation in response to EGF-like growth factors (45). In human breast cancer cells, nuclear localization of ErbB4 correlates with poor survival (46).
Our *in vivo* findings suggest that p14 may play a role in altering the tumorigenic potential of MMTV-infected cells. This characteristic seems to depend on the phosphorylation status of p14, serine 65 being phosphorylated by CK2. Since the CK2\(^{-}\) mutant cells demonstrate impaired tumorigenicity, it is likely that phosphorylation of serine 65 by this kinase is associated with enhanced tumorigenicity. Indeed, CK2, a highly conserved serine/threonine protein kinase that is ubiquitously distributed in eukaryotes, is known to play an important role in promoting tumorigenesis (47). This is the case for a variety of tumors where CK2 inhibitors have been applied in an effort to impair tumor growth. By extension, since the PKC\(^{-}\) mutant cells demonstrate enhanced tumorigenicity, it is possible that phosphorylation of serine 18 of p14 is associated with decreased tumorigenicity. The exact nature of the kinase(s) that phosphorylate(s) serine 18 is not yet defined. Since the double mutant “CK2\(^{-}\)-PKC\(^{-}\)” demonstrates impaired tumorigenicity similar to that of the CK2\(^{-}\) mutant cells, the CK2\(^{-}\) mutation seems to exert a dominant negative effect over the PKC\(^{-}\) mutation in the context of p14. It is thus tempting to speculate that the balance between the phosphorylation of serine 18 (decreased tumorigenicity) and serine 65 (increased tumorigenicity) could determine whether p14 will act in the capacity of a tumorigenic or anti-tumorigenic agent. This could alter the oncogenic potential of MMTV in infected target cells, based on the proportion of CK2 to PKC sites phosphorylated.

Katz et al (13) have previously proposed that the Env protein of MMTV may play a role in mammary tumorigenesis. They demonstrated that ectopic expression of MMTV Env in normal mammary epithelial cells resulted in phenotypic transformation, and an activation motif in this protein (the Immunoreceptor Tyrosine-
based Activation Motif - ITAM) was critical to that activity. Although subsequent studies (using Env expression in transgenic mice) indicated that ITAM is not sufficient for cellular transformation, Env signaling, nevertheless, takes part in MMTV-mediated transformation, as mutation of the ITAM reduces virus-induced mammary tumorigenesis (48, 49). Taken together, our findings, and those of Ross’s group, are consistent with a role for the Env protein and its signal peptide in regulating the tumorigenic potential of MMTV-infected cells.

MMTV-induced mouse mammary tumors develop following the proviral integration into common integration sites. Genes commonly affected by MMTV insertion in multiple individual tumors include members of the Wnt, Fgf, and Rspo gene families, as well as Notch4 and eIF3e (50). Activation of these genes is not always sufficient to induce tumorigenesis and additional events must occur to initiate it (50). We propose that p14 (with, or without ITAM) provides a “missing link” that (following insertional mutagenesis) enhances or impairs (depending on its phosphorylation status) the tumorigenicity of mammary cells infected with MMTV.

The above findings (including transcriptional regulation of cellular genes by p14) suggest a novel strategy whereby retroviruses modulate their host physiology via signal peptides. This modulation may be mediated via nucleolar targeting of such viral signal peptides. In the case of p14, phosphorylation status dictates tumorigenic potential. Thus, at least in the case of MMTV, the signal peptide clearly performs more functions than previously appreciated.

Acknowledgments

This work was supported in part by the Israel Science Foundation: ISF 1308-04 [to JH]. We would like to thank Prof. Uzi Motro of the Hebrew University for statistical
advice, and Dr Yoav Smith of the Hadassah Medical School for the assistance with
the bio-informatic analysis.
References


Legends to Figures

Figure 1. Mutations generated in the amino acid sequence of p14.
A) The first 98 amino acids of the p73 env precursor protein of MMTV is cleaved,
resulting in the MMTV-p14 protein shown. The sequence of gp52 starts at E99, which
is not part of p14. Arrows indicate point mutations and the amino acid substituted.
The point mutations within the NLS are double mutants, in which both indicated basic
amino acids are changed to either histidines, or glutamate and aspartate. Deletions are
indicated by brackets.
B-F) Ectopic and endogenous expression of wt-p14 in MCF-7 and T-67 murine
lymphoma cells (18), respectively. Immunofluorescence (B-E) and Western blot (F)
analyses were performed using α-p14 antibodies as described in Materials and
Methods. B) naïve MCF-7 cells. C) MCF-7 p14-wt cells. D) MCF-7 p14-wt cells in
the presence of DOX. E) T-67 lymphoma cells. F) lane 1 - MCF-7 cells; lane 2 -
MCF-7 p-14wt cells; lane 3 - MCF-7 p-14wt cells in the presence of DOX; lane 4 –
T-67 cells.

Figure 2. Ectopically-expressed NLS-mutated forms of the p14 protein in MCF-7
cells localize progressively less into the nucleolus.
Immunofluorescence was performed as previously described (20).

Figure 3. Co-immunoprecipitation of p14 and B23.
Whole cell extracts of MCF-7 cells expressing wild type or mutant p14 were
immunoprecipitated with α-p14 antibodies (A) or with α-B23 antibodies (B) as
previously described (19) and analyzed by western blot using α-B23 and α-p14
antibodies as indicated.

Figure 4. Mobility of MMTV-p14.
A) MMTV-p14 is highly mobile in the nucleolus. GFP-p14 was stably expressed in
MCF-7 cells and fluorescence recovery after photobleaching (FRAP) was followed as
described in Materials and Methods.
B) Nuclear export of wild-type and mutant MMTV-p14 in mouse-human
heteokaryons.
Human cells (MCF-7) expressing wild-type or mutant p14 as indicated were fused with mouse cells (NIH-3T3) as described in Materials and Methods. From left to right are shown bright field, Hoechst staining and immunofluorescence with \( \alpha \)-p14 antibodies of the same field. Mouse nuclei in heterokaryons are circled.

LMB – leptomycin B.

Figure 5. MMTV-p14 is a functional phosphoprotein affecting tumorigenicity.

A-B) Putative phosphorylation sites on the p14 protein are phosphorylated in vivo and in vitro. A) Whole cell extracts of MCF-7 cells expressing wild type or mutant p14 were immunoprecipitated with \( \alpha \)-p14 antibodies and then analyzed by western blot using \( \alpha \)-phosphoserine antibodies. B) His-tagged p14 (wild type) was expressed in bacteria and purified. Phosphorylation with CK2 and PKC was performed as described in Materials and Methods and the results analyzed by western blot using \( \alpha \)-phosphoserine antibodies.

C) Tumor development after injection of MCF-7 cells expressing wild-type or p14 phosphorylation mutants into mammary fat pads of SCID mice. MCF-7 cells expressing wild-type or mutant p14 were injected bilaterally into mammary fat pads of SCID mice and development of tumors followed as described in Materials and Methods. For statistical analysis, see the text.

Figure 6. Genes differentially expressed in MCF-7 cells expressing p14 PKC- and CK2- mutants.

A) Volcano plot of 4/ANOVA analysis of expression of PKC- vs CK2- cells. To challenge the statistical significance of the microarray results, we used the data obtained with the chips (described above) to perform a Volcano analysis. This revealed 142 genes whose expression changed (either up or down) by a factor of at least 2 \( (P \)-values less than 0.005 calculated using the \( t \)-test). The 18 most statistically significant of these genes are shown in this figure (B & C). The complete list of genes is available in the supplemental data.
B-C) Absolute expression in the four cell lines of B) the 11 genes with increased expression in PKC\(^-\) cells as compared to CK2\(^-\) cells, and C) the 7 genes with increased expression in CK2\(^-\) cells as compared to PKC\(^-\) cells.

D) Change in the expression of L5 and ErbB4 RNA levels as measured using qRT-PCR. RNA was extracted from cells expressing wt, PKC\(^-\), and CK2\(^-\) p14, as well as from naïve MCF-7 cells. qRT-PCR was performed and the results analyzed as described in Materials and Methods. Fold change is shown in relation to RNA extracted from naïve cells and normalized to GAPDH.
Table 1: The 18 genes which changed most significantly in PKC\(^{-}\) vs CK2\(^{-}\) cells.

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<th>Transcript ID</th>
<th>Function</th>
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<tr>
<td>C10orf107</td>
<td>No known function</td>
<td>9.39E-07</td>
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<tr>
<td>SLC36A4</td>
<td>Sodium-independent electroneutral transporter</td>
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<td>WLS</td>
<td>Essential role in WNT-mediated cell-cell communication</td>
<td>6.04E-05</td>
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<td>EDIL3</td>
<td>Inhibits formation of vascular-like structures</td>
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<td>TAGLN</td>
<td>Involved in calcium interactions and contractile properties of the cell</td>
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<td>PCDH10</td>
<td>Potential calcium-dependent cell-adhesion protein</td>
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<td>GABRB3</td>
<td>GABAA receptor contain an integral chloride channel</td>
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<td>PVRL3</td>
<td>Plays a role in cell-cell adhesion</td>
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<td>BMPR1B</td>
<td>Member of the bone morphogenetic protein receptor family</td>
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<td>PEG10</td>
<td>Prevents apoptosis in hepatocellular carcinoma cells</td>
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<td>SGCE</td>
<td>A link between the F-actin cytoskeleton and the extracellular matrix</td>
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<td>ERBB4</td>
<td>Tyrosine-protein kinase, cell surface receptor and EGF family member.</td>
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<td>BMP7</td>
<td>Induces cartilage and bone formation</td>
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<td>PRSS8</td>
<td>Possesses a trypsin-like cleavage specificity</td>
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<td>PCDH17</td>
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<td>PARP8</td>
<td>Catalyzes the addition of multiple ADP-ribose moieties to proteins.</td>
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<td>SOX2</td>
<td>Controls a number of genes involved in embryonic development</td>
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Figure 2
**Figure 3**

(A) Immunoprecipitation (IP) with αP14 followed by Western Blot (WB) with αB23 and αP14.

(B) IP with αB23 followed by WB with αB23 and αP14.
Figure 5
Figure 6
Molecular Cancer Research

The Signal Peptide of Mouse Mammary Tumor Virus-Env: A Phosphoprotein Tumor Modulator

Daphna Feldman, Maayan Roniger, Allan Bar-Sinai, et al.

Mol Cancer Res  Published OnlineFirst June 27, 2012.

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