MMTV Env encodes an ITAM responsible for transformation of mammary epithelial cells in three-dimensional culture

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Expression of immunoreceptor tyrosine–based activation motif (ITAM)–containing signaling proteins is normally restricted to hematopoietic tissues. The basal activity of ITAM-containing proteins is mediated through negative regulation by coreceptors restricted to hematopoietic tissues. We have identified an ITAM signaling domain encoded within the *env* gene of murine mammary tumor virus (MMTV). Three-dimensional structures derived in vitro from murine cells stably transfected with MMTV *env* display a depolarized morphology in comparison with control mammary epithelial cells. This effect is abolished by Y>F substitution within the Env ITAM, as well as inhibitors of Syk and Src protein tyrosine kinases. Env-expressing cells bear hallmarks of cell transformation such as sensitivity to apoptosis induced by tumor necrosis factor (TNF)–related apoptosis–inducing ligand (TRAIL) or TNFα, as well as down-regulation of E-cadherin and Keratin-18. Human normal mammary epithelial cells expressing MMTV Env also develop transformed phenotype, as typified by growth in soft agar and Matrigel invasion. These disruptions are abrogated by Y>F substitutions. We conclude that ITAM-dependent signals are generated through MMTV Env and trigger early hallmarks of transformation of mouse and human mammary epithelial cells. Therefore, these data suggest a heretofore unappreciated potential mechanism for the initiation of breast cancer and identify MMTV Env and ITAM-containing proteins in human breast tumors as probable oncoproteins.

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Abbreviations used: ITAM, immunoreceptor tyrosine–based activation motif; MMTV, murine mammary tumor virus; SU, surface unit; TM, trans-membrane unit; TRAIL, TNF-related apoptosis-inducing ligand.

Immunoreceptor tyrosine–based activation motifs (ITAMs) are signaling motifs associated with activation, survival, and differentiation. ITAM-containing proteins are expressed in many hematopoietic cells, including B and T lymphocytes, mast cells, platelets, and natural killer cells. The amino acid sequence of ITAMs in these tissues is highly conserved. The canonical motif is denoted as: $Yxx(L/I)x_{6-8}Yxx(L/I)$. The tyrosines embedded in this motif are necessary and sufficient for ITAM signaling function. Once phosphorylated by intracellular protein tyrosine kinases of the Src family, the ITAM-associated tyrosines function as docking sites for SH2-containing signaling proteins involved in linking receptor-initiated signals to downstream cellular responses such as proliferation, survival, and differentiation (1).

Our laboratory has recently found that ITAM-containing proteins are able to generate signals independently of the ligand-induced aggregation that is typical of ITAM-containing receptor complexes (2–4). For ligand-independent functions, positioning at the plasma membrane appears necessary and sufficient for signal generation. Normally, in hematopoietic cells, these ligand-independent signals can be constitutive, but maintained at basal levels by a complex balance between Src and Syk family tyrosine kinases and protein tyrosine phosphatases, such as SHP-1 and SHP-2 (5). These phosphatases are localized to the cytoplasm until they are spatially positioned to regions of the plasma membrane occupied by the ITAM-containing proteins by recruitment to hematopoietic-restricted transmembrane proteins with cytoplasmic ITIM (inhibitory) motifs (1). The current model for this signaling would predict that expression of ITAM-containing plasma membrane proteins outside of hematopoietic tissues where this regulation may be absent could lead to deregulated and possibly hyperactive signaling.

Mouse mammary tumor virus (MMTV) is a β-retrovirus that is acquired through milk

and causes adenocarcinomas of adult mammary epithelial tissue. MMTV uses Toll-like receptors 2 and 4 and the transferrin receptor for viral binding and entry (6). The initial targets of infection by MMTV are B and dendritic cells, generally of the Peyer's patches, although T cells can also be infected (7) and transformed (8, 9). In general, MMTV-infected B cells and dendritic cells do not undergo malignant transformation (7, 10). Instead, B cells generate virus particles that, upon transfer to mammary epithelial cells, result in the transformation of these secondary targets (11). In the absence of B cells, the mammary epithelial cells of mice exposed to MMTV are not infected and do not form tumors (12). T cells likely play an important role in the pathogenesis of mammary tumors by providing proliferative signals to infected antigen-presenting cells that present superantigen on their surface (13, 14).

The transforming properties of MMTV have been generally considered to result from positional effects due to proviral integration next to cellular proto-oncogenes. Specifically, cell transformation by MMTV has been attributed to genetic insertion near Wnt/FGF family genes (15). Nevertheless, genetic evidence argues that these events cannot entirely account for the transformation potential of MMTV. In particular, the *gag* and/or *env* gene products of the virus have been linked to transformation in mammary tumor–susceptible mice (16). Furthermore, the incidence and latency of mammary tumor induction in Wnt transgenic mice does not differ substantially from those in MMTV-infected mice (15).

The expression of ITAM-containing proteins has largely been found to be restricted to hematopoietic tissues. However, several oncogenic viruses with tropism for nonhematopoietic cells have been identified and subsequently shown to encode plasma membrane-associated proteins containing canonical ITAMs. These proteins include Epstein Barr virus LMP2A (17), Kaposi's sarcoma virus K1 (18), and bovine leukemia virus gp30 (19), among others. We have identified a canonical ITAM encoded within the *env* gene of MMTV. We show here that expression of the ITAM-containing protein MMTV Env is sufficient to cause morphological changes consistent with transformation in normal mouse and human mammary epithelial cells in vitro. Env expression led to phenotypic changes characteristic of breast cancer in mice and humans in vivo. Specifically, in vitro expression of MMTV Env led to loss of growth arrest, decreased apoptosis, and down-regulated expression of E-cadherin and Keratin-18 in three-dimensional Matrigel cultures. These alterations resulted in profound disruption of the normal polarized acinar structures typical of nontransformed mammary epithelial cells in three-dimensional cultures, as well as increased soft agar colony formation and Matrigel invasion. These studies implicate ITAM-containing proteins as potent oncoproteins. Furthermore, they suggest previously unappreciated roles for viral membrane ITAMs in the selective transformation of nonhematopoietic tissues. Importantly, they argue for a novel mechanism operative in the initiation of mammary gland tumors.

RESULTS

Expression of ITAM–containing MMTV Env leads to depolarization of mammary epithelial acinar structures

The MMTV *env* gene encodes a type-1 membrane glycoprotein that, after proteolytic cleavage, exists as two mature proteins, the surface unit (SU or gp52) and the transmembrane unit (TM or gp36). The sequence, 418-PAYDYAAI-IVKRPPYVLLPVDIGD-441, is located in the surface unit of MMTV Env (Fig. 1 A). Previous genetic studies have not targeted this sequence nor proposed its relevance in etiology of breast epithelial cell transformation in the mouse.

To test whether the MMTV Env and its ITAM could participate in MMTV-mediated transformation, we used three-dimensional cultures of NMuMG murine mammary epithelial cells. Three-dimensional recombinant basement
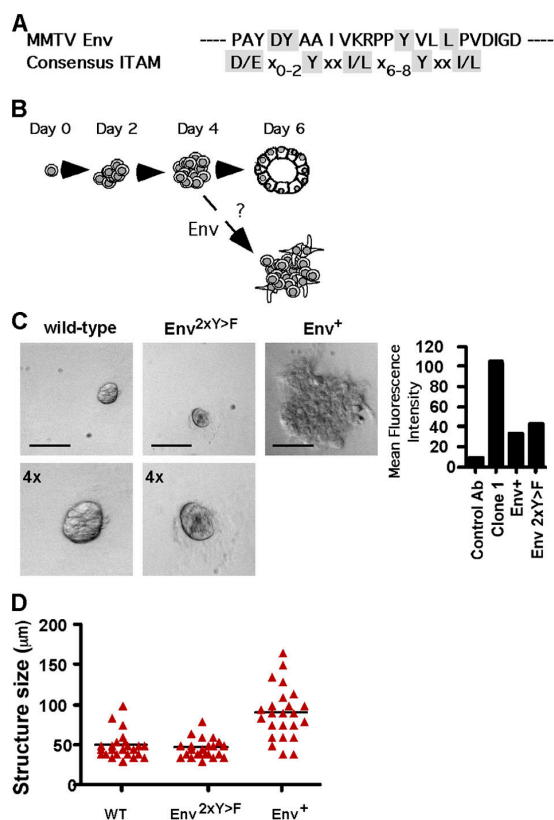

Figure 1. The contribution of ITAM domain in MMTV Env to cell transformation. (A) Schematic comparison of the ITAM in MMTV Env and consensus ITAM sequence. The conserved residues are shaded. (B) Development of mammary epithelial cell acinar structures on Matrigel. The depolarization resulting from MMTV Env expression may lead to loss of the spherical structures. (C) Representative images of three-dimensional cultures of wild-type (WT), mutated (Y^{422}>F, Y^{432}>F) envelope-transfected, envelope-transfected, and MMTV+ NMuMG clone 1 NMuMG cells are shown at day 6 of culture. For WT and F6, the images are also shown at a magnification of 4 of the originals. Note the polarized structures with hollow lumen in both cell types. Bars, 50 μm. (D) Quantification of structure size in a representative experiment (20–60 structures counted for each cell line). The black bars represent the median for each culture. Surface expression of MMTV SU (gp52) is shown for mutated (Y^{422}>F, Y^{432}>F) Env+ and Env+ NMuMG cells. Normal goat IgG was used as the control antibody.

membrane cultures provide a unique opportunity to model the acinar architecture of mammary epithelium in vivo (for review see references 20, 21). Unlike monolayer (two-dimensional) cultures, mammary epithelial cells grown in three-dimensional cultures recapitulate numerous features of breast epithelium in vivo. These include the formation of growth-arrested polarized acini with hollow lumen and deposition of basement membrane components, such as collagen IV and laminin V (22, 23). Three-dimensional cultures provide the appropriate structural and functional context for studying the events involved in morphogenesis of glandular epithelium. Furthermore, three-dimensional cultures provide the opportunity to study the molecular mechanisms necessary to form and maintain a normal glandular architecture and to determine how oncogenes disturb this highly organized structure. Three-dimensional cultures have recently been used in a variety of biological assays, including determining the contribution of ErbB2/HER2 (22), β-integrins (24), and Syk tyrosine kinase (25) to mammary epithelial growth regulation.

We chose the normal murine mammary epithelial cell line NMuMG as our initial model as this cell line has been used in the past to study MMTV-mediated infection and transformation (7, 26). A stably transfected pool, NMuMG.Q4, was established, expressing both the SU and TM subunits of the MMTV Env. Cells from this line, together with mock-transfected NMuMG cells, were seeded in three-dimensional cultures, on a Matrigel cushion. When grown in two-dimensional cultures, these cells did not appear morphologically different from the parental NMuMG cell line and did not show any reproducible growth rate advantage (unpublished data). Nonetheless, depolarization of acinar structure was easily visible in three-dimensional Matrigel cultures (Fig. 1 B). As with other three-dimensional cultures (23, 27), within the first 6 d, WT mock-transfected NMuMG cells were observed to form a polarized disc structure (Fig. 1 C). In this timeframe, morphological differences between mock-transfected cells and Env-expressing cells were readily apparent. Expression of MMTV Env alone was sufficient to generate depolarized acini in frequencies ranging between 30–90% of all structures (nine independent experiments). The differences in depolarization are likely to reflect the variable Env expression levels in the Env$^+$ Q4 line.

For receptor complexes that contain ITAM-containing proteins, signaling is initiated by phosphorylation of the tyrosine residues associated with the ITAM. These phosphotyrosine residues interact with proteins from two families of tyrosine kinases: the Syk/Zap-70 family and the larger Src family (28). Therefore, we determined the contribution of the tyrosine residues in the ITAM domain of Env SU protein. An additional stable transfected pool was generated, NMuMG.F6, that expressed the MMTV Env with two Y>F substitutions (Env$^{2xY>F}$) in the ITAM region of SU (amino acids 422 and 432 in MMTV [C3H] sequence). The Env$^{2xY>F}$ line was almost indistinguishable from wild-type or mock-transfected cells (Fig. 1 C). Occasionally in three-

dimensional cultures of wild-type or Env$^{2xY>F}$ cells, an enlarged structure could be observed, but the frequency of those was always low (six independent experiments; Fig. 1 D) and similar to those observed in mock-transfected NMuMG cells. Differences in surface expression levels of MMTV SU did not account for the observed differences in transformation as expression in the Env-expressing and Env ITAM mutant-expressing lines were equivalent (Fig. 1 B, bottom). Also shown for comparison is the Env expression level on an MMTV-transfected clone (C1), which is higher than on the Env transfectants, verifying that the disruption in the three-dimensional morphology observed is not the result of an artifact of Env overexpression. These results illustrate a striking and unexpected effect of MMTV Env expression on mammary epithelial acinar structures in this in vitro model. Importantly, tyrosine residues within Env were critical for the depolarized morphology of mammary epithelial cells.
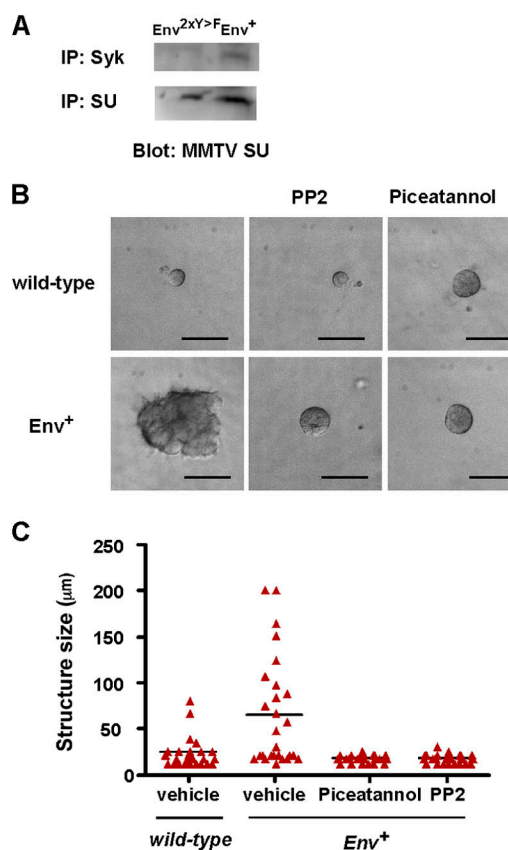

Figure 2. The involvement of Src and Syk kinases in MMTV Env–induced cell transformation. (A) MMTV SU coprecipitates with Syk in a tyrosine-dependent manner. Env$^{2xY>F}$ and Env$^+$ two-dimensional cultures were treated with sodium pervanadate and equal protein loads were used for MMTV SU or Syk immunoprecipitation. A Western blot for MMTV SU is shown. Syk coprecipitates only with Env, but not the 2xY>F mutant (top). (B) Wild-type and Env$^+$ cultures were treated three dimensionally before imaging (at day 6) with either normal assay media, the Src kinase inhibitor PP2 (500 ng/ml), or the Syk kinase inhibitor Piceatannol (500 ng/ml). Bars, 50 μm. (C) Quantification of structure size in a representative experiment (20–60 structures counted for each cell line). The black bars represent the median for each culture.

These alterations are remarkably similar to those induced by known breast oncogenes such as ErbB2/HER2 (22).

Src and Syk tyrosine kinases contribute to MMTV Env–induced acinar depolarization

In lymphocytes, ITAM signaling is dependent on activity of two tyrosine kinase families, the Src family and the smaller Syk/Zap-70 family (1). Constitutive signals stemming from ITAM-containing proteins are of transient nature (5) and their unmasking requires many times the usage of tyrosine phosphatase inhibition (2). Under such conditions, direct interaction between the MMTV SU and Syk kinase can be detected, with up to 34% SU protein coprecipitates with Syk (three independent experiments; Fig. 2 A). In agreement with this observation, pharmacologic inhibitors of the Src (PP2) and Syk/ZAP70 (piceatannol) protein tyrosine kinases are sufficient to block morphological changes associated with Env transformation (Fig. 2 B). Inhibition of either Syk/ZAP70 or Src kinases resulted in the maintenance of the polarized structures. These results were quantified for one representative experiment and are depicted in Fig. 2 C. The inhibitors are each clearly effective in blocking the Env-dependent morphological changes associated with transformation, although there is some variability in the size of the structures.

MMTV Env–expressing cells exhibit transformed mammary epithelial phenotype

We also evaluated NMuMG cells stably expressing infectious MMTV virus (clone 1; reference 26). MMTV[+] clone 1 cells show morphological features in three-dimensional cultures, which resemble mesenchymal cells (Fig. 3 A). A higher level of surface MMTV SU expression, in comparison with the Env-expressing NMuMG line (Fig. 1 C) or positional effects due to virus integration and long-term culture, may account for the greater degree of depolarization observed for this line (Fig. 3 A). These observations prompted us to look for more specific markers of transformation in the three-dimensional cultures of Env[+] and MMTV^{+} cells. First, we examined specific markers of epithelial–mesenchymal transition. Both the Env[+] line and the MMTV[+] clones have down-regulated Keratin-18, suggesting an epithelial–mesenchymal transition as a result of MMTV Env expression (Fig. 3 B and not depicted). Similarly, E-cadherin expression was also observed to be down-regulated in the Env-expressing line and MMTV[+] clone 1 (Fig. 3 B). Expression of E-cadherin and Keratin-18 in NMuMG.F6 (double Y>F envelope mutant) closely resembled wild-type and mock-transfected cells (unpublished data). Interestingly, down-regulation of E-cadherin surface expression can be detected already in Env-expressing cells maintained in two-dimensional cultures (Fig. 3 B, right).

Another hallmark of transformation and depolarization in three-dimensional mammary epithelial cultures is sensitivity to apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL) and TNFα (27). Accordingly, we tested the sensitivity of the Env-expressing and nonexpressing cultures
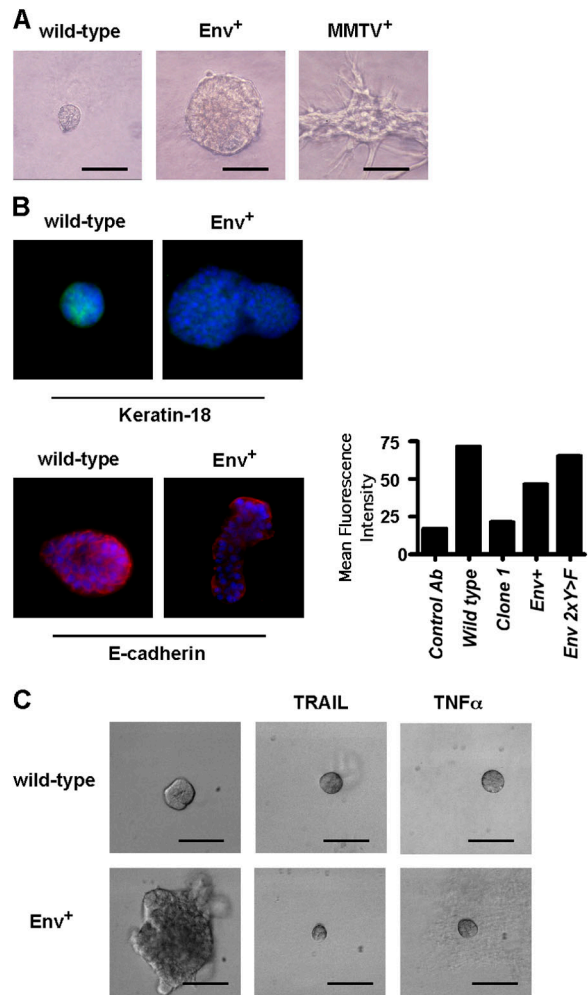

Figure 3. Expression of MMTV Env in murine mammary epithelial cells is sufficient for cell transformation. (A) Representative images of three-dimensional cultures, on Matrigel cushions, of wild-type and Env^{+} NMuMG cells are shown at day 6 of culture. Bars, 50 μm. (B) Keratin-18 staining of mock-transfected, Env[+], and MMTV clone 1 NMuMG cultures at day 6. Keratin-18 is in green and nuclear staining (DAPI) is in blue. (right) E-cadherin surface expression, as quantified by flow cytometry, is reduced in Env[+] and MMTV clone 1 NMuMG two-dimensional cultures. (C) Wild-type and Env[+] cultures were treated 17 h before imaging (at day 6) with either normal assay media, 1 μg/ml TRAIL, or 100 nM TNFα. The induction of apoptosis was confirmed by TUNEL assays (not depicted).

for their sensitivity to these agents. Shown in Fig. 3 C, Env-expressing but not WT or mock-transfected cells were observed to be sensitive to the proapoptotic effects of these agents as determined by their marked attenuated growth and loss of the spreading, nonpolarized structures exhibited by the Env-expressing cells grown in the absence of TNFα or TRAIL. These effects on Env-expressing cells were similar to effects reported previously for mammary epithelial cells in three-dimensional cultures transformed by known mammary oncoproteins (24). Together, the depolarized structures observed in the Env-expressing line and the MMTV[+] NMuMG clones exhibit known markers and hallmarks of mammary cell transformation.
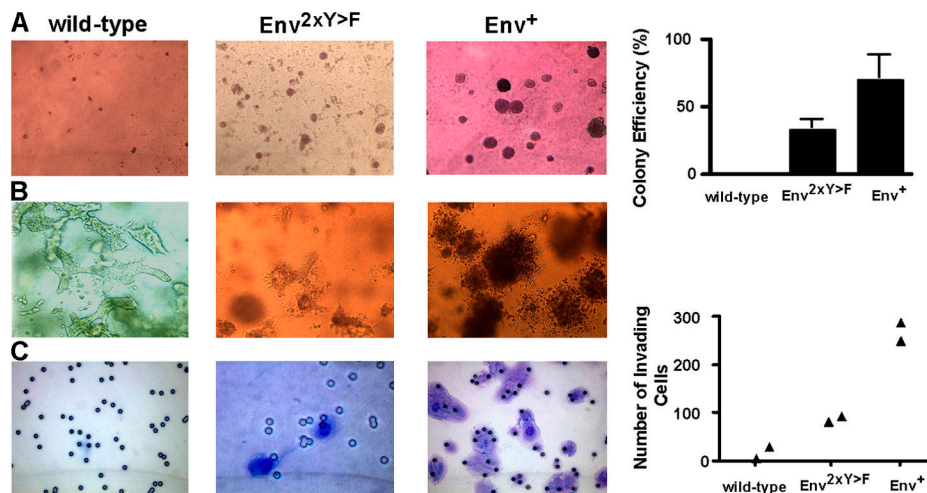
Figure 4. MMTV Env also transforms human mammary epithelial cells. (A) Colony formation assay for wild-type, mutated (Y^{422}>F, Y^{432}>F) Env$^+$ and Env$^+$ MCF-10F cells. Colonies were defined as cell clusters >60 μm in size. (right) The percentage of colonies formed in five agar-methocel cultures in a representative experiment out of three independent experiments. (B) Representative images from collagen-matrix cultures of wild-type, mutated Env$^+$, and Env$^+$ MCF-10F cell lines (a representative experiment out of three independent experiments). Ductal structures are visible in wild-type cultures. Complete loss of ductal structure could be seen in Env$^+$ cultures, whereas mutated Env$^+$ culture exhibit a mixed phenotype. (C) Representative images from invasion assays of wild-type, mutated Env$^+$, and Env$^+$ MCF-10F cells. The three panels on the left are showing the stained mesh after the completion of the invasion assays. Invading cells were visualized by blue stain. The right panel shows total invading cell counts from a representative experiment (in duplicate) out of three independent experiments.

Expression of MMTV Env leads to human mammary epithelial cell transformation

We determined the ability of MMTV Env to transform human mammary epithelial cells, to ascertain whether Env expression leads to phenotypes normally associated with breast malignancy. We generated stable transfectants of the primary human mammary epithelial line, MCF-10F, expressing either WT Env$^+$ (MCF-10F.Q400) or the Env$^{2xY>F}$ envelope mutant (MCF-10F.Y1). As in vitro parameters of cell transformation, we used two assays: colony formation in agar-methocel and three-dimensional growth in collagen matrix (Fig. 4 A). Although both cell lines produced colonies, colony formation in the Env-expressing cells was significantly and reproducibly more efficient (55–90% compared with only 25–40% in Env ITAM mutant-expressing cells). Importantly, the Q400 colonies were approximately twofold larger than those of the ITAM mutant-expressing line. These differences were more evident at earlier time points, where identifiable Env-expressing colonies were detected as early as 5 d in culture.

A second approach to detection of the transforming properties of the Env protein in human cells was to use a collagen matrix assay, where mammary epithelial cells form ductal structures resembling their organization in the mammary gland. In Env-expressing cells, a notable loss of ductal structure was observed in comparison with wild-type MCF-10F cells and the ITAM mutant Env$^+$ (Fig. 4 B). With Env$^+$ cells, only spherical structures could be seen.

Lastly, Matrigel invasion assays were conducted to evaluate the invasive properties Env-expressing human cells. As shown in Fig. 4 C, ITAM mutant Env$^+$ cells were only mildly invasive (<100 cells/25,000 cells seeded), whereas Env$^+$ cells were highly invasive with more than twice as many cells scored in this assay. These studies extend our observations with mouse cells in demonstrating the transforming properties of Env-expression in human mammary epithelial cells.

DISCUSSION

The immune system relies heavily on the function of ITAM-containing proteins. B cells, T cells, mast cells, macrophages, and NK cells all depend on such molecules for their activation and selection processes. The discovery of ITAM-containing viral proteins outside the immune system has led us to investigate what role they could play in a non-hematopoietic context. Previously, viral ITAM-containing proteins were examined almost exclusively within the context of hematopoietic cells and little information existed regarding the functions of ITAM-containing proteins in a nonhematopoietic context. Such studies are important for two main reasons. First, there are clear indications that viral ITAM-containing proteins may play role in a variety of cancers (29, 30). Second, recent studies have shown that cellular ITAM-containing proteins can be expressed outside the immune system (31–33). The function of these proteins is largely unknown.

The data presented demonstrate the ability of the MMTV Env ITAM-containing protein to initiate profound growth and morphological changes that are consistent with malignant transformation of mammary epithelial cells. Furthermore, we show directly that the tyrosine residues in the MMTV SU ITAM and the activation of Syk and Src kinases all play a critical role in the transformation process. We propose that a protein containing an ITAM domain generates a transforming signal in both murine and human normal mammary epi-

thelial cells. The signal generated relies on both Src and Syk tyrosine kinases to exert its transforming capabilities, in a mechanism similar to lymphocyte activation by the B and T cell receptors (34). ITAM-based signaling is well characterized in hematopoietic cells. ITAM-based signaling in hematopoietic cells is tightly regulated by phosphatases, such as SHP-1/2, as part of a negative regulatory loop that exists to terminate the signal (5). It is possible that an analogous termination mechanism does not exist in mammary epithelial cells and that this enables the generation of an unregulated constitutive transforming signal. These studies suggest that MMTV Env may contribute to MMTV-induced transformation. In light of the previous model for MMTV-induced transformation, as reliant solely on positional integration, our findings are remarkable and unexpected.

The investigation of viral ITAM-containing proteins could lead to new insights regarding the way cancer is initiated. Both KSHV K1 and EBV LMP2A were shown to be capable of cell transformation (29, 30). K1 is capable of transforming fibroblasts and ultimately leads to the development of sarcomas (30). Furthermore, it also induces expression of angiogenic and invasion factors critical to cancer, such as vascular endothelial growth factor (35). LMP2A was shown to transform keratinocytes in vitro and tumorigenesis in vivo (29). A few novel cellular ITAM-containing proteins were also identified, such as the SIRP family (32) and the neutrophil-restricted CEACAM3 (36). The function of these proteins is largely unknown. Examination of the behavior of other viral ITAM-containing proteins could also give some insights for a possible association of these sequences in the initiation or progression of mammary epithelial transformation.

The work presented here suggests a new model for mammary epithelial transformation. It is known that ITAM-containing proteins, such as LMP2A, act in two modes of operation: (a) disturbing existing tyrosine-based signaling networks stemming from ITAM-containing complexes such as the B or T cell receptors and (b) generating an independent constitutive signal, which influences the physiology of the host cell (37, 38). Similarly, MMTV Env could integrate other known events in breast cancer tumorigenesis. Both EGFR and β4-integrin signaling are based on tyrosine phosphorylation. The ITAM domain on the SU portion of the envelope protein could act in similar ways to LMP2A and other viral proteins in drawing Src and Syk tyrosine kinases off EGFR or β4-integrin. According to current models, such an alteration in EGF-based signaling could result in an imbalance of both growth and apoptosis (39), leading to the transformed phenotype we have observed (Figs. 1 and 3). Furthermore, manipulation of β4-integrin signaling could induce anchor-independent growth, which is typical of advanced tumorigenesis (27), which we observed in the envelope-expressing human mammary epithelial cells (Fig. 4). Syk tyrosine kinase might play an important role as an integrator of these signals. Syk activity was suggested to be critical for initiation of breast tumorigenesis, and its loss of expression was a marker for more advanced tumors (25).

In this paper, we establish functional relevance of the ITAM in the context of MMTV Env-induced morphological transformation. Like other retroviruses, MMTV encodes an Env polyprotein that is cleaved to generate the gp52 surface (SU) and gp36 subunits. The association of the SU and TM was established more than two decades ago (40–42). In the conventional model for retroviral envelope proteins, the en-
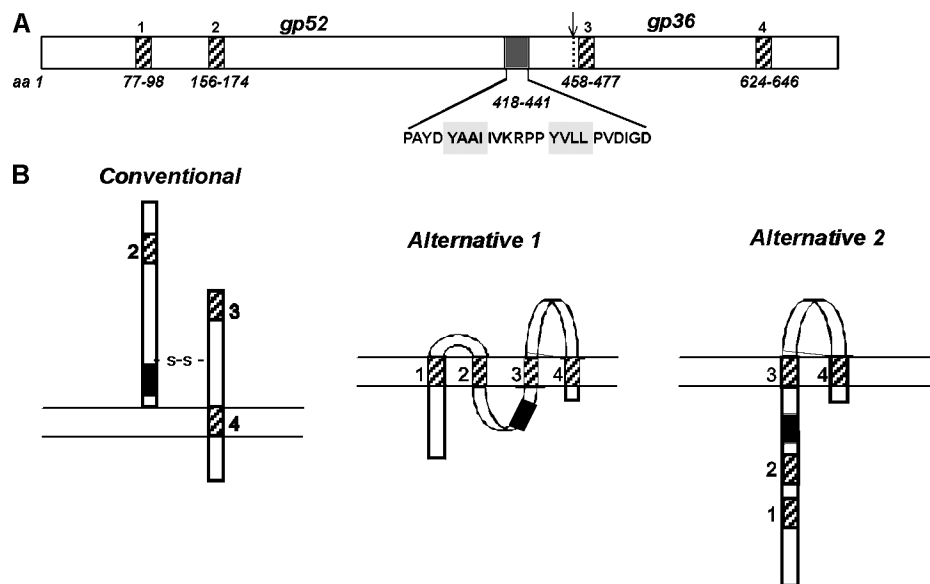

Figure 5.   MMTV envelope sequence, domains, and topological models. (A) Schematic representation of MMTV Env protein and its domains. Potential transmembrane domains are numbered and depicted in diagonally striped boxes. The ITAM region is marked in a shaded box. (B) Models for the surface expression of mature MMTV Env on the host cell surface. The domains are marked as in A. The hydrophobicity scores for the transmembrane domains were as follows: 1, 1,489; 2, 699; 3, 2,658; and 4, 3,134.

tire SU is extracellular (Fig. 5 B). Using algorithms designed to predict transmembrane regions and orientation (TMpred, http://www.ch.embnet.org/software/TMPRED_form.html), we detected four putative transmembrane domains in the MMTV Env protein (Fig. 5, diagonally striped boxes), including one in its unusually long signal peptide (transmembrane domain 1). These observations were confirmed by an additional program PHDhtm, which predicts transmembrane helices (http://www.embl–heidelberg.de/predictprotein/submit_adv.html). Importantly, Platt and Firestone have evidence that transmembrane domain 3, which normally mediates fusion during viral infection, can function as a bona fide transmembrane domain under some circumstances (43). Alternative usage of all transmembrane domains (Fig. 5 B, alternative model 1) or transmembrane domains 3 and 4 (Fig. 5 B, alternative model 2) will result in Env with a cytoplasmic tail including the ITAM region of SU (Fig. 5 A, shaded box). The most likely alternative model is alternative model 2 because domains 3 and 4 are the most hydrophobic domains and are similar in their capability to span the plasma membrane.

Using three standard assays, we showed that Env-expressing human cells were transformed and invasive (Fig. 4). Such phenotype is remarkable because it is typical of mammary epithelial cells with high potential for tumorigenesis and metastasis in humans. Several papers have documented the presence of MMTV homology sequences in ex vivo isolates of human breast epithelium tumors (44–47). These sequences were originally identified by their homology to the Env protein of MMTV. Although this virus has long been known to induce malignant transformation of mouse mammary epithelial cells, a human equivalent of MMTV has only been recently suggested (47), although it is still disputed by many investigators. According to published evidence, ITAM-mediated transformation could operate in a significant proportion of human breast tumors (48). We have generated predicted protein sequences in accordance with MMTV Env homologous sequences published by Pogo et al. (46). Out of 14 amino acid sequences analyzed, 5/11 (45%) breast cancer samples and 1/3 (33%) human mammary cell lines showed considerable homology to MMTV SU, including the putative ITAM motif. Moreover, three other predicted protein sequences for the MMTV Env human homologue also include an intact ITAM domain (GenBank/EMBL/DDBJ accession nos. AAF64164, AAK60129, and AAP73835). In addition, although the surrounding region has some homology to human endogenous retrovirus K, retroviruses of this family do not possess an ITAM sequence (unpublished data).

In summary, signaling through ITAM sequences may play an important part in mammary epithelial transformation. Whether those effects represent early initiation processes or are sufficient for tumorigenesis in vivo remains an issue for further in depth study. Additional studies are also necessary to explore the precise molecular mechanism enabling such rapid and invasive tumors to develop, as the re-sult of the expression of this protein. In the long term, understanding this mechanism will enable a new approach to possible treatments for breast cancer.

MATERIALS AND METHODS

Cell lines. The NMuMG and MCF-10F cell lines were obtained from the American Type Culture Collection. MMTV-transfected clones of the NMuMG cell line, clone 1, were generated as reported previously (26). All NMuMG cell lines were maintained in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10 μg/ml insulin at 37°C and 5% CO_2.

Cell transfections were accomplished using the GenePorter system (Gene Therapy Systems) according to the manufacturer's instructions. The Q61 plasmid was used for complete envelope expression (26). Mutations in the MMTV SU tyrosine residues (Y>F) were introduced using the Quickchange XL kit from Stratagene. Stable pools were generated and maintained by sorting for SU^{hi} expressing cells every 5–10 passages.

Flow cytometry. Cells grown in two-dimensional cultures (10^6) were analyzed for flow cytometry on FACSCalibur (BD Biosciences). Goat polyclonal anti-SU (26) or rat anti–human E-cadherin (Sigma-Aldrich) were used as primary antibody and donkey anti–goat IgG-FITC conjugated or goat anti–rat IgG-Cy5 conjugated were used as secondary antibody (Jackson ImmunoResearch Laboratories). Normal goat IgGs or rat IgGs were used as the negative control.

Three-dimensional cultures. NMuMG cells (5×10^3 cells per chamber) were cultured on Matrigel (BD Biosciences) cushions following the precise protocol published previously (23) in the absence of exogenous EGF. The structures were analyzed, at a magnification of 20, on a Zeiss Axiovert 200M equipped with PCO SensiCam video camera and Slidebook software (Intelligent Imaging Innovations). Cell staining was performed as described previously (23) with rat anti–human Keratin-18 (Lab Vision), rat anti–human E-cadherin and goat anti–rat IgG-FITC conjugated antibodies (Jackson ImmunoResearch Laboratories), or goat anti–rat IgG-Alexa-555 conjugated antibodies (Molecular Probes). Quantification of structure size was done using a 10×50-μm grid reticule (Fisher Scientific) and 20–100 structures were counted from each chamber. The inhibitors PP2 and Piceatannol (EMD) were added on day 3 of culture and pictures were taken on day 6. In apoptosis assays, TNFα (R&D Systems) or TRAIL (BIOMOL Research Laboratories, Inc.) were added on day 5 culture and pictures were taken on day 6. TUNEL assay (Roche) was performed according to the manufacturer's instructions (except that all steps were done in room temperature).

Cell lysis, immunoprecipitation, and Western blotting. Cells grown in two-dimensional cultures to confluency were stimulated with 50 mM sodium pervanadate as described previously (2) and harvested by cell lifters (Sigma-Aldrich). The cell pellet was lysed using PhosphoSafe (EMD), supplemented with protease inhibitor cocktail (Roche), and 0.5% wt/vol sodium azide. Equivalent protein loads were used for immunoprecipitation as determined by Bichinchoninic Acid assay (Sigma-Aldrich). The antibody used for immunoprecipitation was mouse anti-SU black 8–6 monoclonal antibody (10). Immunoprecipitation and Western blotting were performed as described previously (2). For detection, the antibodies used were either goat anti-SU or rabbit anti-Syk N-19 (Santa Cruz Biotechnology, Inc.) polyclonal antibodies. Donkey anti–goat IgG-alkaline phosphatase conjugated antibody or goat anti–rat IgG-alkaline phosphatase conjugated antibody was used as a secondary antibody (Jackson ImmunoResearch Laboratories). For development and quantification, an ECF substrate was used followed by a scan using Storm 860 and analysis by ImageQuant 5.2 (all obtained from Amersham Biosciences).

The in vitro human model of cell transformation. The transforming potential of MMTV envelope protein on human breast epithelial cells in

vitro was evaluated by using the spontaneously immortalized MCF-10F cells. MCF-10F cells and the transfected stable pools derived from them were maintained in DMEM:F-12 [1:1] medium with a 1.05 mM Ca²⁺ concentration. Culture media were prepared by the Central Tissue Culture Facility at the Fox Chase Cancer Center. After transfection, the cells were assayed for determination of survival efficiency, colony efficiency, colony size, ductulogenic capacity, and invasiveness in Boyden chambers.

Colony formation in agar-methocel. This technique was used as an in vitro assay for anchorage-independent growth, a parameter indicative of transformation. All cell lines were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8% methocel (Sigma-Aldrich) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each transfection group were plated in eight 24-well chambers precoated with 0.5 ml 5% agar base in DMEM:F-12 medium. Cells were fed with fresh feeding medium containing 0.8% methocel twice a week. The top four wells were stained with neutral red 1:300 ratio at 24 h after plating, and the total number of viable cells was counted at a magnification of 10. The bottom four wells were maintained in culture for 21 d. After 21 d, these wells were stained with neutral red and the number of colonies was counted. 10 colonies per well were measured by using a graduated reticule under microscope at a magnification of 10. Colony efficiency was determined by a count of the number of colonies >63 μm in diameter and expressed as a percentage of the original number of viable cells after 24 h of plating.

Ductulogenesis in collagen matrix. This in vitro technique evaluates the capacity of cells to differentiate by examining their ability to form three-dimensional structures in a collagen matrix. Parental MCF-10F and transfected cells were suspended at a final density of 2×10^3 cells/ml in 89.3% Vitrogen¹⁰⁰ collagen matrix (Collagen) and plated in four 24-well chambers precoated with 89.3% collagen. The cells were fed with fresh feeding medium containing 20% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 d or longer to determine whether they formed ductal structures or whether they grew as ball-like spherical masses. The structures were photographed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological examination.

Invasion assay. BioCoat Matrigel Invasion Chambers (BD Biosciences) provide cells with the conditions that allow assessment of their invasive property in vitro. The invasion chambers were used according to the manufacturer's instructions. In brief, trypsinized cells (2.5×10^4) were seeded in the top chamber and incubated for 22 h at 37°C. High calcium medium with 20% horse serum was used as a chemoattractant. The filters were fixed, stained by Diff Quick (Sigma-Aldrich), cut out, and mounted onto glass slides. The total number of cells that crossed the membrane was counted under a light microscope.

REFERENCES

1. Harder, T., and K.R. Engelhardt. 2004. Membrane domains in lymphocytes—from lipid rafts to protein scaffolds. *Traffic*. 5:265–275.
2. Bannish, G., E.M. Fuentes-Panana, J.C. Cambier, W.S. Pear, and J.G. Monroe. 2001. Ligand-independent role for the B lymphocyte antigen receptor and their role in positive selection during B lymphopoiesis. *J. Exp. Med.* 194:1583–1596.
3. Fuentes-Panana, E.M., G. Bannish, and J.G. Monroe. 2004. Basal B-cell receptor signaling in B lymphocytes: mechanisms of regulation and role in positive selection, differentiation, and peripheral survival. *Immunol. Rev.* 197:26–40.
4. Fuentes-Panana, E.M., G. Bannish, N. Shah, and J.G. Monroe. 2004. Basal Igalpha/Igbeta signals trigger the coordinated initiation of pre-B cell antigen receptor-dependent processes. *J. Immunol.* 173:1000–1011.
5. Monroe, J.G. 2004. Ligand-independent tonic signaling in B-cell receptor function. *Curr. Opin. Immunol.* 16:288–295.
6. Zhang, Y., J.C. Rassa, M.E. deObaldia, L.M. Albritton, and S.R. Ross. 2003. Identification of the receptor binding domain of the mouse mammary tumor virus envelope protein. *J. Virol.* 77:10468–10478.
7. Dzuris, J.L., T.V. Golovkina, and S.R. Ross. 1997. Both T and B cells shed infectious mouse mammary tumor virus. *J. Virol.* 71:6044–6048.
8. Dudley, J.P., A. Arfsten, C.L. Hsu, C. Kozak, and R. Risser. 1986. Molecular cloning and characterization of mouse mammary tumor proviruses from a T-cell lymphoma. *J. Virol.* 57:385–388.
9. Rajan, L., C.A. Kozak, and J.P. Dudley. 1998. Chromosomal localization of acquired MMTV proviral integration sites in T-cell lymphomas. *Mamm. Genome.* 9:84–85.
10. Burzyn, D., J.C. Rassa, D. Kim, I. Nepomnaschy, S.R. Ross, and I. Piazzon. 2004. Toll-like receptor 4-dependent activation of dendritic cells by a retrovirus. *J. Virol.* 78:576–584.
11. Wellinger, R.J., M. Garcia, A. Vessaz, and H. Diggelmann. 1986. Exogenous mouse mammary tumor virus proviral DNA isolated from a kidney adenocarcinoma cell line contains alterations in the U3 region of the long terminal repeat. *J. Virol.* 60:1–11.
12. Golovkina, T.V., J.P. Dudley, and S.R. Ross. 1998. B and T cells are required for mouse mammary tumor virus spread within the mammary gland. *J. Immunol.* 161:2375–2382.
13. Choi, Y., J.W. Kappler, and P. Marrack. 1991. A superantigen encoded in the open reading frame of the 3′ long terminal repeat of mouse mammary tumour virus. *Nature*. 350:203–207.
14. Golovkina, T.V., A. Chervonsky, J.P. Dudley, and S.R. Ross. 1992. Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *Cell*. 69:637–645.
15. Callahan, R., and G.H. Smith. 2000. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. *Oncogene*. 19:992–1001.
16. Hook, L.M., Y. Agafonova, S.R. Ross, S.J. Turner, and T.V. Golovkina. 2000. Genetics of mouse mammary tumor virus-induced mammary tumors: linkage of tumor induction to the gag gene. *J. Virol.* 74:8876–8883.
17. Caldwell, R.G., J.B. Wilson, S.J. Anderson, and R. Longnecker. 1998. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity*. 9:405–411.
18. Lee, H., J. Guo, M. Li, J.K. Choi, M. DeMaria, M. Rosenzweig, and J.U. Jung. 1998. Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. *Mol. Cell. Biol.* 18:5219–5228.
19. Willems, L., J.S. Gatot, M. Mammerickx, D. Portetelle, A. Burny, P. Kerkhofs, and R. Kettmann. 1995. The YXXL signalling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads. *J. Virol.* 69:4137–4141.
20. Park, C.C., M.J. Bissell, and M.H. Barcellos-Hoff. 2000. The influence of the microenvironment on the malignant phenotype. *Mol. Med. Today*. 6:324–329.
21. Jacks, T., and R.A. Weinberg. 2002. Taking the study of cancer cell survival to a new dimension. *Cell*. 111:923–925.
22. Muthuswamy, S.K., D. Li, S. Lelievre, M.J. Bissell, and J.S. Brugge. 2001. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat. Cell Biol.* 3:785–792.
23. Debnath, J., K.R. Mills, N.L. Collins, M.J. Reginato, S.K. Muthuswamy, and J.S. Brugge. 2002. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell*. 111:29–40.

24. Weaver, V.M., O.W. Petersen, F. Wang, C.A. Larabell, P. Briand, C. Damsky, and M.J. Bissell. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J. Cell Biol*. 137:231–245.

25. Coopman, P.J., M.T. Do, M. Barth, E.T. Bowden, A.J. Hayes, E. Basyuk, J.K. Blancato, P.R. Vezza, S.W. McLeskey, P.H. Mangeat, and S.C. Mueller. 2000. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature*. 406:742–747.

26. Dzuris, J.L., W. Zhu, D. Kapkov, T.V. Golovkina, and S.R. Ross. 1999. Expression of mouse mammary tumor virus envelope protein does not prevent superinfection in vivo or in vitro. *Virology*. 263:418–426.

27. Weaver, V.M., S. Lelievre, J.N. Lakins, M.A. Chrenek, J.C. Jones, F. Giancotti, Z. Werb, and M.J. Bissell. 2002. beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell*. 2:205–216.

28. Songyang, Z., S.E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X.R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, et al. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol*. 14:2777–2785.

29. Scholle, F., K.M. Bendt, and N. Raab-Traub. 2000. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. *J. Virol*. 74:10681–10689.

30. Lee, H., R. Veazey, K. Williams, M. Li, J. Guo, F. Neipel, B. Fleckenstein, A. Lackner, R.C. Desrosiers, and J.U. Jung. 1998. Deregulation of cell growth by the K1 gene of Kaposi's sarcoma-associated herpesvirus. *Nat. Med*. 4:435–440.

31. Tomasello, E., C. Cant, H.J. Buhring, F. Vely, P. Andre, M. Seiffert, A. Ullrich, and E. Vivier. 2000. Association of signal-regulatory proteins beta with KARAP/DAP-12. *Eur. J. Immunol*. 30:2147–2156.

32. Kharitonenkov, A., Z. Chen, I. Sures, H. Wang, J. Schilling, and A. Ullrich. 1997. A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature*. 386:181–186.

33. Ilan, N., and J.A. Madri. 2003. PECAM-1: old friend, new partners. *Curr. Opin. Cell Biol*. 15:515–524.

34. Kurosaki, T. 2002. Regulation of B cell fates by BCR signaling components. *Curr. Opin. Immunol*. 14:341–347.

35. Wang, L., N. Wakisaka, C.C. Tomlinson, S.M. DeWire, S. Krall, J.S. Pagano, and B. Damania. 2004. The Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) K1 protein induces expression of angiogenic and invasion factors. *Cancer Res*. 64:2774–2781.

36. McCaw, S.E., J. Schneider, E.H. Liao, W. Zimmermann, and S.D. Gray-Owen. 2003. Immunoreceptor tyrosine-based activation motif phosphorylation during engulfment of *Neisseria gonorrhoeae* by the neutrophil-restricted CEACAM3 (CD66d) receptor. *Mol. Microbiol*. 49:623–637.

37. Fruehling, S., and R. Longnecker. 1997. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR–mediated signal transduction. *Virology*. 235:241–251.

38. Dykstra, M.L., R. Longnecker, and S.K. Pierce. 2001. Epstein-Barr virus coopts lipid rafts to block the signaling and antigen transport functions of the BCR. *Immunity*. 14:57–67.

39. Rosfjord, E.C., and R.B. Dickson. 1999. Growth factors, apoptosis, and survival of mammary epithelial cells. *J. Mammary Gland Biol. Neoplasia*. 4:229–237.

40. Racevskis, J., and N.H. Sarkar. 1980. Murine mammary tumor virus structural protein interactions: formation of oligomeric complexes with cleavable cross-linking agents. *J. Virol*. 35:937–948.

41. Dion, A.S., A.A. Pomenti, and D.C. Farwell. 1979. Vicinal relationships between the major structural proteins of murine mammary tumor virus. *Virology*. 96:249–257.

42. Callis, A.H., and E.M. Ritzi. 1980. Detection and characterization of mouse mammary tumor virus cell surface antigens: estimation of antigen abundance by protein A assay. *J. Virol*. 35:876–887.

43. Platt, E.J., and G.L. Firestone. 1991. Expression of mouse mammary tumor virus glycoprotein truncations defines roles for the transmembrane domain and ectodomain hydrophobic region in constitutive exocytic trafficking and proteolytic processing. *J. Biol. Chem*. 266:19384–19395.

44. Fitzgerald, K.A., E.M. Palsson-McDermott, A.G. Bowie, C.A. Jefferies, A.S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M.T. Harte, et al. 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature*. 413:78–83.

45. Ford, C.E., D. Tran, Y. Deng, V.T. Ta, W.D. Rawlinson, and J.S. Lawson. 2003. Mouse mammary tumor virus-like gene sequences in breast tumors of Australian and Vietnamese women. *Clin. Cancer Res*. 9:1118–1120.

46. Wang, Y., I. Pelisson, S.M. Melana, V. Go, J.F. Holland, and B.G. Pogo. 2001. MMTV-like env gene sequences in human breast cancer. *Arch. Virol*. 146:171–180.

47. Xu, L., Z. Shen, L. Guo, B. Fodera, A. Keogh, R. Joplin, B. O'Donnell, J. Aitken, W. Carman, J. Neuberger, and A. Mason. 2003. Does a betaretrovirus infection trigger primary biliary cirrhosis? *Proc. Natl. Acad. Sci. USA*. 100:8454–8459.

48. Wang, Y., J.F. Holland, I.J. Bleiweiss, S. Melana, X. Liu, I. Pelisson, A. Cantarella, K. Stellrecht, S. Mani, and B.G. Pogo. 1995. Detection of mammary tumor virus env gene-like sequences in human breast cancer. *Cancer Res*. 55:5173–5179.