Oncogenicity of AKR Mink Cell Focus-Inducing Murine Leukemia Virus Correlates with Induction of Chronic Phosphatidylinositol Signal Transduction

AHMAD M. AL-SALAMEH AND MILES W. CLOYD*

Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

Received 23 January 1992/Accepted 21 July 1992

Naturally occurring recombinant murine leukemia viruses (MuLVs), termed mink cell focus-inducing (MCF) viruses, are the proximal leukemogens in spontaneous thymic lymphomas of AKR mice. The mechanism by which these viruses transform lymphocytes is not clear. Previous studies have implicated either integrational activation of proto-oncogenes, chronic autocrine immune stimulation, and/or autocrine stimulation of growth factor receptors (e.g., interleukin 2 receptors) via binding of the viral *env* glycoprotein (gp70) to these receptors. Any one of these events could also involve activation of second messenger signaling pathways in the cell. We examined whether infection with oncogenic AKR-247 MCF MuLV induced transmembrane signaling cascades in thymocytes of AKR mice. Cyclic AMP levels were not changed, but there was enhanced turnover of phosphatidylinositol phosphates, with concomitant increases in diacylglycerol and inositol 1,4,5-triphosphate. Thus, phospholipase C activity was increased. Protein kinase C activity was also elevated in comparison to that in uninfected thymocytes. The above events occurred in parallel with MCF expression in the thymus and were chronically maintained thereafter. No changes in phospholipid turnover occurred in an organ which did not replicate the MCF virus (spleen) or in thymocytes of AKR mice infected with a thymotropic, nononcogenic MCF virus (AKV-1-C36). Therefore, only the oncogenic MCF virus induced phosphatidylinositol signal transduction. Flow cytometric comparison of cell surface gp70 revealed that AKR-247 MCF virus-infected thymocytes expressed more MCF virus gp70 than did thymocytes from AKV-1-C36 MCF virus-infected mice, suggesting that certain threshold quantities of MCF virus *env* glycoproteins may be involved in this signaling. This type of signal transduction is not induced by stimulation of the interleukin 2 receptor but is involved in certain oncogene systems (e.g., *ras* and *fms*). Its chronic induction by oncogenic MCF MuLV may thus initiate thymocyte transformation.

Certain types of naturally occurring recombinant murine leukemia viruses (MuLVs), termed mink cell focus-inducing (MCF) viruses, are the proximal leukemogens in the genesis of spontaneous lymphomas in AKR mice (13, 23, 41). Since MuLVs do not possess transduced cellular proto-oncogenes, the mechanism(s) by which they transform cells is not clear. The most popular theory regarding the mechanism of transformation by leukemia viruses postulates that the virus activates latent cellular proto-oncogenes by integration into nearby cellular DNA (24). It is consistent with this hypothesis that MuLV-induced leukemias are typically monoclonal with regard to the integration site of the proviruses in the host chromosome, and preferred integration regions have been observed in several MuLV systems (14, 15, 44, 48). In AKR MCF MuLV-induced lymphomas, 10 to 20% of the tumors have viral integrations near *c-myc* when examined early after appearance (34, 40), and up to 65% of late-developing lymphomas in this system were shown to have MCF virus integrations near *c-myc* (40). However, 0 of 14 spontaneous AKR lymphomas possessed proviral integrants near *c-myc* (51). Although these studies show that integration near a known proto-oncogene can be frequent, they do not prove that it is required for tumor development. A certain percentage of lymphomas do not contain retroviral integrations near known oncogenes. Furthermore, in most cases it has been difficult to prove that proviral activation of a single gene is sufficient to initiate and maintain a tumor,

and many of the proto-oncogenes activated are not oncogenic by themselves (58). Several studies have indicated that proto-oncogenic activation may be due to late events which contribute increased malignant properties during neoplastic progression (17, 21, 43, 53, 54).

Another theory of MuLV transformation centers around the fact that the cells transformed are cells of the immune system, and it states that MuLVs may antigenically stimulate the infected lymphocytes in a chronic autocrine fashion, leading to lymphoma induction (31, 38).

A third theory focuses on MCF virus envelope glycoprotein, gp70, binding to and stimulating certain types of growth factor receptors, rather than antigen receptors. Recent reports by Li et al. (33) and Li and Baltimore (32) showed that spleen focus-forming virus (SFFV) or Friend and Moloney MCF virus gp70 bound to the erythropoietin receptor when coexpressed in transfected cells. This resulted in induction of factor-independent proliferation of factor-dependent cells (32, 33). Thus, this and the previous theory center on gp70 binding with cell surface receptors as a critical element in MCF virus transformation. Currently, it is not known to which receptors AKR MCF virus gp70 binds on thymocytes. Only a small subpopulation of thymocytes express erythropoietin-related interleukin 2 (IL-2) receptors (10), while a large percentage of cortical thymocytes become infected with MCF virus (unpublished data). Thus, further evidence is needed that MCF virus gp70 binds to specific activation or growth receptors (T-cell receptors or IL-2 receptors) on the thymocyte surface and that this binding is involved in transformation.

* Corresponding author.

Finally, two additional mechanisms could be theoretically important for MuLV to induce T-cell lymphomas in rodents: proto-oncogene cooperation and oncogenic mutations other than those due to provirus integration. Although there is little evidence implicating these mechanisms in MCF virus leukemogenesis, cooperating proto-oncogenes or mutated proto-oncogenes can usurp normal growth or differentiation pathways (1, 4, 5, 55).

A number of features of MCF MuLVs have been correlated with their oncogenicity. There is good correlation between oncogenic potential and the amount of virus (and thus virus protein) that is produced in the infected organ (13). Genetic studies revealed that all of the MCF viral genes were involved in its oncogenicity (25), which suggested that oncogenicity depended, at least minimally, on the virus's being able to efficiently infect and replicate in the target cells. MuLV envelope proteins, which localize to the cell membrane, have been shown to be particularly important in oncogenicity of at least one type of MCF MuLV (SFFV) (35, 60). Because of this, and because transformation activity of some oncogenes (e.g., *ras* and *fms*) (4) is mediated at the cell membrane, we theorized that MCF MuLVs may, in effect, have "oncogenic" proteins which also act at the membrane. Whether MuLV transformation is mediated through binding of viral proteins to cell surface receptors or is directly mediated via insertion of these proteins in the membrane is not known. Therefore, we examined whether AKR MCF MuLV infection induced signaling pathways of the cell membrane, and if so, what types of signal cascades were involved. The latter information could also help to reveal whether receptors are involved and, if so, which ones.

MATERIALS AND METHODS

Mice and cells. AKR/Cum mice (1 to 5 days old) were inoculated intraperitoneally with 10^4 to 10^5 focus-forming units of either AKR-247 MCF or AKV-1-C36 MCF virus. Uninoculated littermates were used as controls. Thymocytes were aseptically removed from the mice at 3, 4, 5, 6, 7, and 8 weeks after injection, and the cells were dispersed in RPMI 1640 medium containing 10% fetal bovine serum at a concentration of 10^7 cells per ml.

cAMP extraction and assay. Thymocytes (5×10^6) were washed twice with ice-cold phosphate-buffered saline (PBS), homogenized in ethanol-1 N HCl (100:1 [vol/vol]), and allowed to stand for 5 min at room temperature for the protein to coagulate. Following centrifugation, the supernatant was collected, and the precipitate was washed with 1 ml of ethanol- H_2O (2:1) and centrifuged. The supernatants were combined and evaporated to dryness. Cyclic AMP (cAMP) was assayed in the supernatant by using an Amersham cAMP radioimmunoassay kit as described by the manufacturer.

Phospholipid labeling, extraction, and separation. $^{32}P_i$ (ICN Radiochemical) was added to the thymocytes at 25 μ Ci/ml of medium and incubated for 6 h at 37°C. Medium was removed from the cells, and the incubation was stopped by rinsing the cells twice with cold PBS. Phospholipids were extracted from the cells in methanol-chloroform-2.4 N HCl (100:100:1 [vol/vol/vol]; containing 0.05 mg of butylated hydroxytoluene per ml as an antioxidant), 0.25 ml of 10 mM EDTA was added, and the tubes were vortexed. The resulting two phases were centrifuged at $2,000 \times g$ for 5 min, and the lower chloroform phase was transferred to a clean test tube. The upper aqueous phase was treated with 0.2 ml of chloroform, mixed, and centrifuged, and the organic phase was trans-

ferred to the tube containing the original organic phase. Next, 1 ml of methanol-2.4 N HCl (100:1 [vol/vol]) was added to the combined chloroform phases, the contents of the tubes were mixed together, and the final chloroform phase, with the purified lipids, was separated from the aqueous phases by centrifugation. Chloroform was removed from the lipids by evaporation under N_2 .

The phospholipids were separated by thin-layer chromatography (TLC) on Whatman K6F silica gel 60A plates. The dried lipids were reconstituted in 50 μ l of chloroform-methanol (1:1 [vol/vol]), and then the lipids were spotted onto TLC plates. The plates were developed in a solvent of chloroform-methanol- NH_4OH - H_2O (90:70:6:14 [vol/vol]); by a modified procedure of Gonzalez-Sastre and Folch-Pi [22]). The positions of the radiolabeled lipids on the plates were determined by autoradiography with X-ray film. Spots on the silica gel containing radioactive lipids were scraped from the TLC plates into scintillation vials, and the radioactivity was measured with a liquid scintillation spectrometer.

Labeling and extraction of inositol phosphates. [^3H]myo-inositol (15 μ Ci/ml) was added to freshly dispersed thymocytes, and the cells were incubated for 20 h at 37°C. LiCl was then added (10 mM), and the cells were incubated for an additional 2 h at 37°C. Cells were then centrifuged, rinsed twice with medium, and lysed in 0.5 ml of 5% (wt/vol) ice-cold trichloroacetic acid. The samples were neutralized with 100 mM EDTA and 1 ml of 1,1,2-trichlorotrifluoroethane-tri-*n*-octylamine (50:50 [vol/vol]) and thoroughly mixed, and the two lower phases were removed by aspiration. The remaining aqueous samples containing the inositol phosphates were separated by anion-exchange high-pressure liquid chromatography (HPLC; 10- μ m Partisil Sax [250 by 4.6 mm column]; Alltech). The inositol phosphates were eluted with 45 ml of a linear gradient (0 to 1.5 M) of ammonium formate-phosphoric acid (pH 3.7), started after 10 ml of distilled water was passed through the column, at a flow rate of 1.25 ml/min. The column was further calibrated with [^3H]inositol 1-phosphate (IP_1), [^3H]inositol 1,4-diphosphate (IP_2), and [^3H]inositol 1,4,5-triphosphate (IP_3) (Amersham). Fractions were collected, and ^3H radioactivity was quantitated by liquid scintillation counting.

Labeling and extraction of diacylglycerol (DAG). [^3H]oleic acid (50 μ Ci/ml) (Amersham) was added to the thymocytes, which were then incubated for 6 h at 37°C. The cells were rinsed twice with PBS, lysed with 0.5 ml of chloroform-methanol (1:1 [vol/vol]), containing 0.05 mg of butylated hydroxytoluene per ml as an antioxidant, mixed, and centrifuged. The samples were transferred to clean test tubes, dried under nitrogen, and redissolved in 50 μ l of chloroform-methanol (2:1 [vol/vol]). Neutral lipids were separated by TLC by using Whatman K6F silica gel 60A plates, with chloroform-methanol-ether-acetic acid (75:140:45:15 [vol/vol/vol/vol]) as the developing solvent. Standard mixtures containing 1,2-DAG, 1,3-DAG, monoglyceride, triglyceride, free fatty acids, and cholesterol esters were run in parallel lanes. Following development, the plates were air dried, and then the lipid spots were visualized by exposure to iodine vapor for 2 h. The spots were then scraped into scintillation vials and quantitated by scintillation counting.

MCF virus quantitation. Freshly dispersed thymocytes (5×10^5) were incubated with 100 μ l of monoclonal antibody (H514) for 1 h at room temperature. Cells were washed twice with Hanks balanced salt solution (HBSS) containing 2% calf serum. Fluorescein isothiocyanate-conjugated secondary antibody (100 μ l) (anti-mouse immunoglobulin M) (1:50 dilution) was added to the cells, and the cells were incubated

for 1 h at room temperature, washed twice with HBSS, and preserved in 100 μ l of PBS containing 2% formalin. Cells were analyzed on an EPICS profile flow cytometer (Coulter), and the percentage and fluorescence intensity of positive cells (cells expressing gp70 on their membrane) were determined.

PKC assay. Thymocytes (5×10^7) were washed twice with ice-cold PBS, suspended in buffer (20 mM Tris-HCl, pH 7.5, with 2 mM EDTA and 50 mM β -mercaptoethanol), and homogenized by sonication, as described elsewhere (37). The homogenate was then centrifuged at $100,000 \times g$ at 4°C for 60 min. The supernatant was saved for the analysis of cytosolic protein kinase C (PKC) activity. The pellet was resuspended in ice-cold homogenization buffer [20 mM Tris-HCl, pH 7.5, with 2 mM EDTA, 50 mM β -mercaptoethanol, 10 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), and 0.1% Triton X-100], stirred for 30 min at 4°C , and then centrifuged at $100,000 \times g$ at 4°C for 30 min. The supernatant was used for the assay of PKC in the particulate (membrane) fraction. The standard assay mixture (200 μ l, final volume) contained 20 mM Tris-HCl (pH 7.5), 5 mM Mg acetate, 0.5 mM CaCl_2 , 40 μ g of histone type IIS, 10 μ M ATP (1 μ Ci of [γ - ^{32}P]ATP per assay), and the enzyme (cytosolic or particulate fraction). CaCl_2 (2.1 mM) was added to the assay mixture of the particulate fraction to neutralize the effect of EGTA in the homogenization buffer. The control (blank sample) did not contain 0.5 mM CaCl_2 , 4 μ g of phosphatidylserine, and 0.1 μ g of diolein, and instead, 0.5 mM EGTA was added to the mixture. Incubation was carried out at 37°C for 10 min. Reactions were terminated by the addition of 1 ml of 25% ice-cold trichloroacetic acid. Trichloroacetic acid-precipitated material was collected and washed with 10% trichloroacetic acid on a Millipore filter (0.45- μ m pore size, type BA85; Schleicher & Schuell) by vacuum filtration. Filters were dried and placed in scintillation vials, and the radioactivity was determined by liquid scintillation counting.

RESULTS

Relative quantities of MCF virus envelope glycoproteins expressed on thymocytes infected with oncogenic and nononcogenic MCF viruses. Earlier studies demonstrated a correlation between oncogenicity of AKR MCF virus and its efficiency of infection and replication in thymocytes (13, 41). While these viral properties could enhance the probability of a rare integration event near a proto-oncogene, they would also result in an increased quantity of viral proteins produced per infected cell. Hypothesizing that AKR MCF virus may initiate thymocyte transformation via effects of its *env* glycoproteins on the thymocyte membrane, we compared the relative amounts of MCF virus gp70 expressed on the surfaces of thymocytes from mice infected with oncogenic versus nononcogenic MCF viruses, using immunofluorescence intensity measurements on a flow cytometer. Following inoculation of baby AKR mice with AKR-247 (oncogenic) or AKV-1-C36 (nononcogenic) MCF virus, thymocytes isolated from both of these mice were stained with MCF virus *env*-specific monoclonal antibody (H514) at 2, 4, and 6 weeks postinfection (Fig. 1; Table 1). H514 mean fluorescence intensity on thymocytes from mice inoculated with AKR-247 MCF virus was greater than that for AKV-1-C36 MCF virus-infected mice, showing that, on average, more MCF virus gp70 was expressed on the thymocyte cell surfaces of AKR-247 MCF virus-infected mice (Fig. 1). At 4 to 6 weeks postinfection, 35 to 38% of total thymocytes in AKR-247 MCF virus-infected mice expressed MCF virus

FIG. 1. Immunofluorescence flow cytometric analysis of MCF virus envelope glycoprotein (gp70) on thymocyte membranes at 4 weeks postinfection. Cells were incubated with MCF virus *env*-specific monoclonal antibody (H514) for 1 h, washed, then labeled with secondary antibody fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin M, and washed. The cells were then analyzed with an EPICS profile flow cytometer equipped with a 3-W laser. (A) Thymocytes from AKR-247 MCF virus-infected mice stained with isotype control primary monoclonal antibody (immunoglobulin M); (B) uninfected thymocytes stained with anti-MCF gp70 (H514) (control); (C) thymocytes infected with AKR-247 MCF virus stained with H514; (D) thymocytes infected with AKV-1-C36 MCF virus stained with H514. Data are representative of four experiments.

gp70, while 15 to 23% of AKV-1-C36 thymocytes expressed MCF virus gp70 (Table 1). These results show that AKR-247 MCF virus replicates in approximately twice the number of thymocytes and, on average, expresses more gp70 per cell than does AKV-1-C36 MCF virus. Previous infectious center tests demonstrated that 10- to 100-fold more MCF virus is produced by AKR-247 MCF-infected thymocytes than by AKV-1-C36 MCF virus-infected thymocytes (13). Thus, the

TABLE 1. Relative expression of MCF virus gp70 on thymocytes from AKR mice infected with nononcogenic and oncogenic MCF viruses^a

Week post-infection	% Thymocytes positive		Mean fluorescence	
	AKR-247	AKV-1-C36	AKR-247	AKV-1-C36
2	0	0	1.4	1.5
4	34.4	14.7	37.3	28.0
6	37.9	22.8	38.2	30.6

^a Immunofluorescence staining was done for MCF virus envelope glycoprotein gp70 on thymocyte membranes. Freshly harvested thymic cells were stained with monoclonal antibody (H514), followed by secondary antibody fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin M, and were analyzed by flow cytometry. The data are presented as the percentage of cells that express gp70 on the membrane and as the relative mean fluorescence intensity.

FIG. 2. Effect of MCF viral infection on thymocyte cAMP levels. Mice were injected intraperitoneally with AKR-247 or AKV-1-C36 MCF virus. Thymocytes were isolated at 7 and 9 weeks postinfection. Cells were homogenized in ethanol-1 N HCl (100:1) and washed with ethanol-H₂O (2:1 [vol/vol]). cAMP was determined by radioimmunoassay (Amersham) and expressed as picomoles per 10⁷ thymocytes.

amount of MCF virus or MCF virus proteins produced correlates with the oncogenicity of the virus.

MCF virus infection does not change cellular cAMP levels. If MCF virus infection/replication stimulates membrane signaling processes, then changes should be observed in components of these cascades. We first examined and compared

the levels of cAMP in thymocytes from infected and uninfected mice. Thymocytes from mice infected with AKR-247 MCF virus 7 and 9 weeks previously, as well as from age-matched control (uninfected) mice, were removed, and the quantity of cAMP was determined by using a commercial cAMP radioimmunoassay. As another control, cAMP levels were also determined in thymocytes from AKR mice infected with nononcogenic AKV-1-C36 MCF virus. The cAMP levels in all samples tested were approximately the same (Fig. 2), demonstrating that the cAMP second messenger pathway was not changed by MCF virus infection.

MCF virus infection stimulates phosphoinositide cleavage.

We next examined the components of the phosphoinositide signaling pathways. A key event of signal transduction through that pathway is the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to give IP₃ and DAG (2, 36). When IP₃ is produced following receptor stimulation by a ligand, this is unequivocal evidence that PIP₂ is the lipid used by the receptor mechanism. That finding does not exclude, however, the possibility that other inositol lipids are also being hydrolyzed. It has been suggested that PIP₂ is used at the beginning of the signaling cascade but is followed later by a calcium-dependent hydrolysis of phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PIP) (59).

An example of the phospholipid changes observed and a summary of these changes over time in thymocytes of AKR-247 MCF virus-infected mice is shown in Fig. 3. At 4 weeks postinfection, there was a reduction in the amount of ³²P-labeled PIP and PIP₂ in the thymocytes infected with AKR-247 MCF virus compared with thymocytes from unin-

FIG. 3. Steady-state phospholipid levels in MCF virus-infected thymocytes. (A) Autoradiogram of TLC-separated phospholipids extracted from thymocytes of AKR/Cum mice at 6 weeks postinfection with thymotropic, oncogenic AKR-247 MCF MuLV and of age-matched control (uninfected) mice. Thymocytes (10⁷) were incubated with ³²P_i (50 μCi/ml) for 6 h. The cells were rinsed, lipids were extracted and separated by thin-layer chromatography with chloroform-methanol-ammonia-H₂O, and the plate was subjected to autoradiography. Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPC, lysophosphatidylcholine; PG, phosphatidylglycerol; PC, phosphatidylcholine. (B) Summary of the levels (expressed as the percentage of control [uninfected] thymocytes) of labeled phospholipids in thymocytes of AKR/Cum mice at various times postinfection with AKR-247 MCF MuLV. Data from three or four individual mice are summarized for each time point.

FIG. 4. Lack of phospholipid changes in organs in which MCF MuLV did not replicate. Autoradiogram of TLC-separated phospholipids extracted from metabolically labeled spleen cells from AKR/Cum mice uninoculated or inoculated 5 weeks previously with AKR-247 MCF MuLV. This autoradiogram is representative of these experiments performed with three different mice, all of which showed that no changes occurred.

ected, age-matched, control mice. After the radioactivity of individual TLC phospholipid spots had been determined by scintillation counting, about 45% of radiolabeled PIP_2 and 20% of PIP were cleaved; this was possibly phospholipase C ($\text{PLC-}\gamma$) mediated. The low level of PIP_2 was maintained throughout the time course of infection, and the level of PIP was decreased further to 45% of that of the controls by the sixth, seventh, and eight weeks postinfection. There were no changes in the quantities of phosphatidylcholine or other phospholipids, including PI plus phosphatidylserine (PS). We could not measure the concentrations of PI in infected thymocytes because it comigrated with phosphatidylserine in our TLC system. Furthermore, it would be difficult to detect small changes in the PI pool, which was at least 8- to 10-fold larger than the polyphosphoinositide pools.

The changes observed paralleled expression of the MCF virus. The decreases in the concentrations of PIP and PIP_2 were not due to decreased ^{32}P uptake, since we observed no decreases in ^{32}P labeling of phosphatidylcholine or other phospholipids in infected cells. Thus, replication of AKR-247 MCF virus in thymocytes activated what appeared to be a typical PI signal transduction, but in a chronic manner. The observation that there were no changes in lysophosphatidylcholine also indicated that phospholipase A_2 activity was not affected.

As further controls, we examined the phospholipid changes in spleen cells of the same mice. The virus does not replicate in these cells (12). No changes were observed in phospholipid turnover in spleen cells (see Fig. 4 for a representative example); this observation further indicated that the phospholipid changes in the thymocytes were induced by the replicating MCF virus.

Do phospholipid changes occur in thymocytes in which nononcogenic AKV-1-C36 MCF virus does not replicate?

FIG. 5. Steady-state phospholipid levels in thymocytes of AKR/Cum mice at various times postinfection with the thymotropic nononcogenic AKV-1-C36 MCF virus. Thymi were removed from infected and age-matched uninfected mice; cells were dispersed and labeled with ^{32}P ; and phospholipids were extracted and analyzed by TLC. Individual spots were scraped and quantified by liquid scintillation counting, and the data for the MCF virus-infected mice are presented as the percentage of the results for control (uninfected) mice.

Thymocytes infected with and showing replication of the thymotropic, but nononcogenic, MCF MuLV (AKV-1-C36) were also examined to determine whether PI signaling changes were induced by any MCF virus or only by oncogenic ones. Thymocytes from these mice were removed at the same times postinfection as above and labeled with ^{32}P for phospholipid analysis. No changes in phosphoinositides occurred in these cells (summarized in Fig. 5). Approximately 15 to 23% of the thymocytes showed replication of the MCF virus (see Table 1), which was enough for detecting phospholipid changes if they occurred. In addition, there were no changes in phosphatidylcholine or other phospholipids. These results thus associate PI changes with replication of an oncogenic MCF virus.

The phosphoinositide changes associated with oncogenic MCF virus replication represent true signal transduction. $\text{PLC-}\gamma$ -mediated hydrolysis of PIP_2 will yield increased quantities of DAG and IP_3 . We thus examined whether AKR-247 MCF virus infection changed DAG and IP_3 levels in the thymocytes as a measure of activation of PIP_2 -specific $\text{PLC-}\gamma$. Thymocytes from mice inoculated with AKR-247 MCF virus were metabolically labeled in vitro with ^3H myo-inositol for 20 h, and H_2O -soluble lipids were extracted and analyzed by HPLC. Figure 6 shows that IP_3 levels in infected thymocytes were raised to about twofold that of the control. This was performed for three different mice, with the same results. It should be noted that LiCl (10 mM) was included so that inositol phosphate would accumulate and not be recycled into phospholipid (3). We also measured DAG turnover and found DAG levels to be two- to threefold that of uninfected thymocytes (Fig. 7). This was the case from the third to the eighth week postinfection. Therefore, AKR-247 MCF virus replication appeared to chronically activate $\text{PLC-}\gamma$.

MCF virus infection activates PKC. In the canonical PI signaling cascade, the next step following $\text{PLC-}\gamma$ cleavage of polyphosphoinositides is the activation of PKC. DAG, in conjunction with Ca^{2+} , is an endogenous activator of PKC and causes intracellular translocation of PKC from the cytosol to the membrane. We examined the activities of PKC in the membrane and cytosolic fractions of MCF virus-infected thymocytes (Fig. 8). As expected from the

FIG. 6. Representative HPLC elution profile of inositol phosphates in AKR-247 MCF virus-infected thymocytes. [^3H]inositol phosphates from metabolically labeled AKR-247 MCF virus-infected and uninfected thymocytes at 4 weeks postinfection were analyzed by HPLC anion-exchange chromatography. The inositol phosphates were eluted with a 45-ml linear gradient of 0 to 1.5 M ammonium formate-phosphoric acid (pH 3.7), started after 10 ml of distilled water was passed through the column (flow rate of 1.25 ml/min). The column was calibrated with [^3H]inositol 1-phosphate, [^3H]inositol 1,4-diphosphate, and [^3H]IP $_3$. Fractions were collected, and ^3H radioactivity was quantitated by liquid scintillation counting. Two other experiments gave similar results.

increased levels of DAG and IP $_3$ in thymocytes in which AKR-247 MCF virus replicated, membrane-associated PKC activity was about 175% of that of uninfected thymocytes, whereas in AKV-1-C36 MCF virus-infected thymocytes, membrane PKC activity was only 117% of the activity in the uninfected cells. The former value correlates with the extent of elevation of the steady-state levels of DAG and IP $_3$ in the AKR-247 MCF virus-infected thymocytes.

DISCUSSION

This study demonstrated that replication of an oncogenic MCF MuLV in AKR thymocytes induced the canonical PI signal transduction cascade involving activation of PLC- γ , leading to enhanced hydrolysis of PIP and PIP $_2$, elevation of

intracellular levels of DAG and IPs, and activation of PKC. This pathway is known to activate many cellular events, including changes in intracellular ion concentrations and transcription of certain genes. PKC has been shown to activate the NF- κB , *c-rel*, and *c-jun* transcription factors (6–8, 22), as well as *c-ras* (16). Normal activation of PI signal transduction events by hormones or ligands is acute, lasting for only seconds to minutes (52). The chronic signal transduction that occurs in oncogenic MCF virus-infected thymocytes is abnormal and, in essence, leads to chronic activation of PKC in a manner similar to that of the tumor-promoting phorbol esters (39, 49).

A number of oncogene proteins are also known to act at various steps of this signaling cascade. The *v-sis* product, which is a homolog of the platelet-derived growth factor β

FIG. 7. DAG levels in thymocytes infected with the oncogenic AKR-247 MCF virus. Cells were labeled to isotropic equilibrium with [^3H]oleic acid for 6 h, and total lipids were extracted and separated by TLC. The lipid spots were visualized by exposure to iodine vapor for 2 h, and the spots, identified by standards that were also run on the plate, were quantified by being scraped into scintillation vials and counted on a scintillation counter.

FIG. 8. Activation of PKC by AKR-247 MCF virus. The activities of PKC in the cytosolic and membrane fractions from thymocytes of AKR/Cum mice infected with AKR-247 or AKV-1-C36 MCF virus were assayed by determining the incorporation of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP into histone H1 in a cell-free system (see Materials and Methods). CNTRL, control. Bars represent standard error of the mean.

chain, transforms fibroblasts by activating endogenous platelet-derived growth factor receptors (57). This, in turn, causes recruitment of pp74^{c-raf}, PI-3-kinase, *ras* GAP, and PLC- γ (9). v-ErbB oncoprotein is homologous to the epidermal growth factor receptor and has been shown to signal through enhanced phospholipid metabolism (29). Likewise, both the *c-fms* and *v-fms* products have been shown to activate PLC- γ (27, 50). Transformation by the *ras* products, which are homologous to G proteins and possess GTP binding and GTPase activities, has been shown to be accompanied by stimulation of PLC-mediated inositol phospholipid metabolism (26, 30). Of course, these are only a few events, among many, which occur during transformation, and the complete biochemical mechanisms of neoplastic transformation in these systems are not yet defined.

Our finding that MCF virus chronically activates PI signaling adds to earlier theories of the mechanism of leukemia virus transformation. Weissman and McGrath hypothesized that T lymphocytes express specific antigen receptors for certain oncogenic MuLVs and that infection by one of these viruses would specifically bind to and stimulate clonal expansion of specific T cells (38). It was speculated, then, that infection with virus resulted in a chronic autocrine antigenic stimulation leading to neoplasia. Lee and Ihle proposed a similar model which did not involve specific receptors for the oncogenic viruses but rather involved specific immune receptors for gp70 of nononcogenic ecotropic MuLV on T lymphocytes (31). It was speculated that this would establish a situation in which chronic immune activation by the nononcogenic MuLVs would predispose T lymphocytes to malignant transformation. More direct evidence that MuLV *env* glycoprotein may be oncogenic came from studies of Friend SFFV (a truncated MCF MuLV), which showed that mutations in *env* reduced SFFV oncogenicity (35) and that SFFV gp55 solely expressed in mice induced erythroproliferation (11, 60). Recently, studies by Li et al. have indicated that MCF envelope glycoproteins bind to receptors of the erythropoietin receptor family and have shown that coexpression of either erythropoietin receptor or IL-2 β -chain receptor with MCF virus gp70 caused increased proliferation of fibroblasts or IL-3 independence of an IL-3-dependent B-cell line (32, 33). Likewise, Tschlis and Bear (56) showed that infection by MCF virus conferred IL-2 independence on an IL-2-dependent rat T-cell lymphoma line. Our studies demonstrating that oncogenic MCF virus does induce signal transduction in thymocytes shed more light on the mechanism of transformation by MCF MuLV. Activation of T lymphocytes by the T-cell receptor activates a tyrosine kinase which leads to activation of PLC and increased PKC activity (28). Also, stimulation of T cells by mitogens activates PI signal cascades in a manner similar to that observed in this study (46). Stimulation of the IL-2 receptor, however, does not activate PLC- γ and does not signal through a PI signaling cascade. Rather, it activates another phospholipase which cleaves a phosphoinositol glycan, and it signals in a manner similar to that of insulin and colony-stimulating factor 1 (18, 19). Therefore, our results suggest that MCF MuLV may activate thymocytes through receptors different from the IL-2 receptors or in addition to them.

Further theoretical consideration of the mechanism of MCF virus transformation of thymocytes should probably take into account the fact that MCF virus transformation does increase *c-myc* expression, irrespective of whether provirus is integrated near it, but there is not an increase in the number of cells entering into the cell cycle in the thymus for several weeks following full MCF virus expression (42,

45). It would thus appear that MCF virus infection does not act as a stimulus for cell division, as a growth factor would. Rather, it is possible that MCF virus replication may deter the normal events of thymocyte hematopoiesis, which involves a number of different signals at various stages of differentiation. There are a number of leukemogenic systems which appear to be initiated in this manner (47).

ACKNOWLEDGMENTS

We thank Johnny Peterson for the use of HPLC equipment and Jon Teng for technical advice.

These studies were supported by a grant from the John Sealy Memorial Endowment. A.A.-S. was supported by a James W. McLaughlin Predoctoral Fellowship.

REFERENCES

1. Armelin, H. A., M. C. S. Armelin, K. Kelly, T. Stewart, P. Leder, B. H. Cochran, and C. Stiles. 1984. Functional role for *c-myc* in mitogenic response to platelet-derived growth factor. *Nature (London)* 310:655-660.
2. Berridge, M. J. 1984. Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* 220:345-360.
3. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* 212:473-482.
4. Bishop, J. M. 1991. Molecular themes in oncogenesis. *Cell* 64:235-248.
5. Bos, J. L. 1989. *ras* oncogenes in human cancer: a review. *Cancer Res.* 49:4682-4689.
6. Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist. 1990. Cloning of a mitogen-inducible gene encoding a κ B DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. *Nature (London)* 348:76-80.
7. Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter. 1991. Activation of protein kinase C decreases phosphorylation of *c-jun* at sites that negatively regulate its DNA-binding activity. *Cell* 64:573-584.
8. Bull, P., T. Hunter, and I. M. Verma. 1989. Transcriptional induction of the murine *c-rel* gene with serum and phorbol-12-myristate-13-acetate in fibroblasts. *Mol. Cell. Biol.* 9:5239-5243.
9. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Grazini, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281-302.
10. Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. McDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (London)* 314:98-100.
11. Chung, S.-W., L. Wolf, and S. K. Ruscetti. 1989. Transmembrane domain of the envelope gene of a polycythemia-inducing retrovirus determines erythropoietin-independent growth. *Proc. Natl. Acad. Sci. USA* 86:7957-7960.
12. Cloyd, M. W. 1983. Characterization of target cells for MCF viruses in AKR mice. *Cell* 32:217-225.
13. Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* 151:542-552.
14. Corcoran, L. M., J. M. Adams, A. R. Dunn, and S. Cory. 1984. Murine T lymphomas in which the cellular *myc* oncogene has been activated by retroviral insertion. *Cell* 37:113-122.
15. Cuyper, H. T., G. Selten, W. Quint, M. Zijlstra, E. Robanus-Maandag, W. Boelens, P. van Wezenbeek, C. Melief, and A. Berns. 1984. Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* 37:141-150.
16. Downward, J., J. Graves, P. H. Warne, S. Rayter, and D. A. Cantrell. 1990. Stimulation of p21^{ras} upon T-cell activation. *Nature (London)* 346:719-723.
17. Drinkwater, N. R. 1990. Experimental models and biological mechanisms for tumor production. *Cancer Cells* 2:8-15.
18. Eardley, D. D., and M. E. Koshland. 1991. Glycosylphosphatidylinositol: a candidate system for interleukin-2 signal trans-

- duction. *Science* 251:78–81.
19. Farese, R. V., J. S. Davis, D. E. Barnes, M. L. Standaert, J. S. Babischkin, R. Hock, N. K. Rosic, and R. J. Pollet. 1985. The de novo phospholipid effect of insulin is associated with increases in diacylglycerol, but not inositol phosphates or cytosolic Ca^{2+} . *Biochem. J.* 231:269–278.
  20. Fearon, E. R., and B. Vogelstein. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759–767.
  21. Ghosh, S., and D. Baltimore. 1990. Activation in vitro of NF- κ B by phosphorylation of its inhibitor I κ B. *Nature (London)* 344: 678–682.
  22. Gonzalez-Sastre, F., and J. Folch-Pi. 1968. Thin-layer chromatography of the phosphoinositides. *J. Lipid Res.* 9:532–533.
  23. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. USA* 74:789–792.
  24. Hayward, W. S., B. G. Neel, and S. M. Astrin. 1981. Activation of a cellular *Onc* gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature (London)* 290:475–480.
  25. Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins. 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF-247 in AKR mice. *J. Virol.* 53:158–165.
  26. Huang, M., K. Chida, N. Kamat, K. Nose, M. Kato, Y. Homma, T. Takenawa, and T. Kuroki. 1988. Enhancement of inositol phospholipid metabolism and activation of protein kinase C in ras-transformed rat fibroblast. *J. Biol. Chem.* 263:17975–17980.
  27. Jackowski, S., C. W. Rettenmier, C. J. Sherr, and C. O. Rock. 1986. A guanine nucleotide-dependent phosphatidylinositol 4,5-diphosphate phospholipase C in cells transformed by the v-fms and v-fes oncogenes. *J. Biol. Chem.* 261:4978–4985.
  28. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591–1599.
  29. Kato, M., S. Kawai, and T. Takenawa. 1987. Altered signal transduction in erb-transformed cells. *J. Biol. Chem.* 262:5695–5704.
  30. Lacal, J. C., P. T. Fleming, B. S. Warren, P. M. Blumberg, and S. A. Aaronson. 1987. Involvement of functional protein kinase C in the mitogenic response to the H-ras oncogene product. *Mol. Cell. Biol.* 7:4146–4149.
  31. Lee, J. C., and J. N. Ihle. 1980. Mechanisms of C-type viral leukemogenesis. Correlation of in vitro lymphocytes to viremia and leukemia. *J. Immunol.* 123:2351–2358.
  32. Li, J. P., and D. Baltimore. 1991. Mechanisms of leukemogenesis induced by mink cell focus-forming murine leukemia viruses. *J. Virol.* 65:2408–2414.
  33. Li, J. P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. *Nature (London)* 343:762–764.
  34. Li, Y., C. A. Holland, J. W. Hartley, and N. Hopkins. 1984. Viral integrations near c-myc in 10–20% of MCF 247-induced AKR lymphomas. *Proc. Natl. Acad. Sci. USA* 81:6808–6811.
  35. Machida, C. A., R. K. Bestwick, and D. Kabat. 1984. Reduced leukemogenicity caused by mutations in the membrane glycoprotein gene of Rauscher spleen focus-forming virus. *J. Virol.* 49:394–402.
  36. Majerus, P. W., E. J. Neufeld, and D. B. Wilson. 1984. Production of phosphoinositide-derived messengers. *Cell* 37:701–703.
  37. Malhotra, R. K., S. V. Bhawe, T. D. Wakade, and A. R. Wakade. 1988. Protein kinase C of sympathetic neuronal membrane is activated by phorbol ester—correlation between transmitter release, $^{45}\text{Ca}^{2+}$ uptake, and the enzyme activity. *J. Neurochem.* 51:967–974.
  38. McGrath, M. S., and I. L. Weissman. 1979. AKR leukemogenesis: identification and biological significance of thymic lymphoma receptors for AKR retroviruses. *Cell* 17:65–75.
  39. Nishizuka, Y. 1984. Turnover of inositol phospholipids and signal transduction. *Science* 225:1365–1370.
  40. O'Donnell, P. V., E. Fleissner, H. Lonial, C. F. Koehne, and A. Reicin. 1985. Early clonality and high-frequency proviral integration into the c-myc locus in AKR leukemias. *J. Virol.* 55:500–503.
  41. O'Donnell, P. V., E. Stockert, Y. Obata, and L. J. Old. 1981. Leukemogenic properties of AKR dualtropic (MCF) viruses: amplification of murine leukemia virus-related antigens on thymocytes and acceleration of leukemia development in AKR mice. *Virology* 112:548–563.
  42. O'Donnell, P. V., and F. Traganos. 1986. Changes in thymocyte proliferation at different stages of viral leukemogenesis in AKR mice. *J. Immunol.* 136:720–727.
  43. O'Donnell, P. V., R. Woller, and A. Chu. 1984. Stages in development of mink cell focus-inducing (MCF) virus-accelerated leukemia in AKR mice. *J. Exp. Med.* 160:914–934.
  44. Quint, W., W. Quax, H. van der Putten, and A. Berns. 1981. Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. *J. Virol.* 39:1–10.
  45. Reicin, A., J.-Q. Yang, K. B. Marcu, E. Fleissner, C. F. Koehne, and P. V. O'Donnell. 1986. Deregulation of the c-myc oncogene in virus-induced thymic lymphomas of AKR/J mice. *Mol. Cell. Biol.* 6:4088–4092.
  46. Rozenburg, E. 1986. Early signals in the mitogenic response. *Science* 234:161–166.
  47. Sawyers, C. L., C. T. Denny, and O. N. Witte. 1991. Leukemia and the disruption of normal hematopoiesis. *Cell* 64:337–350.
  48. Selten, G., H. T. Cuypers, M. Zijlstra, S. Melief, and A. Burns. 1984. Involvement of c-myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. *EMBO J.* 131:3215–3222.
  49. Sharkey, N. A., K. L. Leach, and P. M. Blumberg. 1984. Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci. USA* 81:607–610.
  50. Sherr, C. J., C. W. Rettenmier, R. Sacca, M. F. Roussel, A. T. Look, and E. R. Stanley. 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41:655–676.
  51. Steffen, D. 1984. Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. *Proc. Natl. Acad. Sci. USA* 81:2097–2101.
  52. Storey, D. J., S. B. Shears, C. J. Kirk, and R. H. Michell. 1984. Stepwise enzymatic dephosphorylation of inositol 1,4,5-triphosphate to inositol in liver. *Nature (London)* 312:374–376.
  53. Sukumar, S. 1969. Ras oncogenes in chemical carcinogenesis. *Curr. Top. Microbiol. Immunol.* 148:93–114.
  54. Thomassen, D. G., T. M. Gilmer, L. A. Annab, and J. C. Barrett. 1985. Evidence for multiple steps in neoplastic transformation of normal and preneoplastic Syrian hamster embryo cells following transfection with harvey murine sarcoma virus oncogene (v-Ha-ras). *Cancer Res.* 45:726–733.
  55. Travalì, S., K. Reiss, A. Ferber, S. Petralia, W. E. Mercer, B. Calabretta, and R. Baserga. 1991. Constitutively expressed c-myc abrogates the requirement for insulinlike growth factor 1 in 3T3 fibroblasts. *Mol. Cell. Biol.* 11:731–736.
  56. Tschlis, P. N., and S. E. Bear. 1991. Infection by mink cell focus-forming viruses confers interleukin 2 (IL-2) independence to an IL-2-dependent rat T-cell lymphoma line. *Proc. Natl. Acad. Sci. USA* 88:4611–4615.
  57. Waterfield, M. D., G. T. Scrase, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel. 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. *Nature (London)* 304:35–39.
  58. Weinberg, R. A. 1985. The action of oncogenes in the cytoplasm and nucleus. *Science* 230:770–776.
  59. Wilson, D. B., T. M. Connolly, T. E. Bross, P. W. Majerus, W. R. Sherman, A. M. Tyler, L. J. Rubin, and J. E. Brown. 1985. Isolation and characterization of the inositol cyclic phosphate products of polyphosphoinositide cleavage by phospholipase C. Physiological effects in permeabilized platelets and Limulus photoreceptor cells. *J. Biol. Chem.* 260:1046–1051.
  60. Wolff, L., and S. K. Ruscetti. 1985. Malignant transformation of erythroid cells in vivo by introduction of a nonreplicating retrovirus vector. *Science* 228:1549–1552.