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Expression of *abl* and other oncogenes is independent of metastatic potential in Abelson virus-transformed malignant murine large cell lymphoma

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The role of oncogene expression in tumor metastasis was examined using the Abelson leukemia virus-transformed murine large cell lymphoma RAW117. Cell sublines of low and high metastatic potential expressed equally *abl* oncogene-coded mRNA and its phosphoprotein product p160, and the capacity of p160 to become autophosphorylated with γ-[^{32}P]ATP was the same among low and high metastatic cells. The expression of other oncogene-coded mRNAs (*fos*, *myc*, *myb*), if present, was also similar in low and high metastatic RAW117 cells. Although oncogene expression is thought to be important in initiating, and in some cases maintaining, the transformed phenotype, its expression in RAW117 lymphoma cells appears to be unrelated to metastatic phenotype.

Introduction

The spread of malignant tumors from primary to near and distant secondary sites occurs via a complex series of sequential steps [19, 34, 35, 44, 45]. At each step of the metastatic process certain tumor cell characteristics are required [34, 38–40, 44], and maintenance of these characteristics necessitates the expression of certain genes that programme for or control metastasis-associated gene products [36, 37, 43].

The role of viral oncogenes or their cellular prototypes in the control of metastatic behavior is largely unknown, although oncogenes appear to be important in the maintenance of the transformed phenotype in some cells [5, 10, 14]. Insight into how cellular processes involved in transformation might be controlled by certain oncogenes has come from studies in which a cellular oncogene was activated by insertion of a viral gene promoter at a DNA site upstream from the cellular oncogene [24]. Other mechanisms of activation of cellular oncogenes, such as point mutation [16, 53], rearrangement [7, 18, 25] and amplification [2, 13] could individually or collectively affect the malignant potential of tumor cells.

We have examined the expression of the *abl* and other oncogenes in murine large cell lymphoma cells transformed by Abelson leukemia virus (AbLV) [46]. Using parental AbLV-transformed RAW117 lymphoma cells (RAW117-P), highly meta-

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static sublines have been established by sequential selection, such as the subline RAW117-H10 selected ten times *in vivo* for liver colonization [6]. RAW117-H10 subline forms 200 to 250 times more gross liver tumor nodules than the RAW117-P line within two weeks after intravenous or subcutaneous injection of tumor cells [48].

Materials and methods

Cells

RAW117 cells were grown and maintained in suspension cultures in Dulbecco-modified Eagle's medium (DME, Gibco, Grant Island, NY, U.S.A.) containing 10 per cent fetal bovine serum (FBS, Reheis Division, Armour Pharmaceutical, Kankakee, IL, U.S.A.). Cell cultures were used within 10 passages from frozen stocks of low passage cells to eliminate possible phenotypic drift in metastatic properties [41]. Cultures were tested for the presence of *Mycoplasma* using Hoechst 33258 staining [8] and were found to be negative.

Animals and metastasis assays

RAW117 sublines were assayed for organ colonization by intravenous injection of $5–10 \times 10^3$ viable tumor cells in 0·1 ml Hank's balanced salt solution into groups of 10 female Balb/c mice [6]. Animals were fed on a diet of chow without silicon dioxide, and were given spring water (chlorine < 1 p.p.m.) *ad libitum*.

Oncogene-encoded mRNA

Total RNA was prepared according to Auffray and Rougeon [3] or Glisin *et al.* [22]. Polyadenylated mRNA was selected by oligo(dT)-cellulose chromatography [4]. Aliquots of 5 μg polyadenylated (pA^+) or nonadenylated (pA^-) mRNA from RAW117 cells were heated for 10 min at 60°C in 50 per cent formamide, 6 per cent formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7·0. The samples were electrophoresed in a 1 per cent agarose gel containing 6 per cent formaldehyde. The RNA was transferred onto a nitrocellulose sheet [54] and hybridized to nick-translated oncogene plasmid inserts [51]. Oncogenes probed included *abl* [29], *fos* [11], *myb* [20], *myc* [12] and *B-lym* [17]. Hybridization was for 16 h at 43°C in 50 per cent formamide, 5X Denhart's solution [15], 5X SCC and 20 mM sodium phosphate, 100 μg salmon sperm DNA, 10 per cent dextran sulfate, pH 7·0 [56]. Hybridized filters were washed at 60°C with 0·1 × SCC and 0·1 per cent sodium dodecyl sulfate (SDS), and autoradiograms were prepared. Molecular weight markers were [^{32}P]-labeled 18S and 28S ribosomal RNA.

abl-oncogene phosphoprotein p160 and Moloney virus products

Tumor cells (10^7) were washed several times by centrifugation and resuspended in PBS. The cells were suspended in 1·5 ml DME (without methionine) containing 250 μCi [^{35}S]methionine (Amersham, U.K.) and 10 per cent dialysed, heat-inactivated FBS, and were incubated for 1 h at 37°C. The cells were then washed by centrifugation and resuspension into PBS, and extracted with 5 ml lysis buffer (10 mM sodium phosphate, 100 mM sodium chloride, 1 per cent Triton X-100, 0·5 per cent SDS, pH 7·5). Cell lysates were treated with non-immune serum and absorbed with *Staphylococcus aureus* [26]. Equivalent amounts of radioactive protein were immunoprecipitated with various antibody preparations, absorbed with *S. aureus*, and separated by polyacrylamide slab gel electrophoresis in SDS [30].

Antibody preparations Ab-T1 and Ab-T2 were made against AbLV-transformed L12 tumor cells and obtained as described previously [59].

Phosphorylation experiments were conducted using immunoprecipitated complexes from unlabeled cell lysates. The immunoprecipitates were collected with *S. aureus*, washed by centrifugation and resuspended in 10 mM magnesium chloride containing 1 μCi γ-[^{32}P]ATP, and incubated for 20 min at 0°C. The complexes were then washed in the same buffer without isotope and subjected to SDS-polyacrylamide slab gel electrophoresis as described [9, 60].

Immunoprecipitation of Moloney leukemia virus (MoLV) gene products was accomplished with anti-MoLV. Cells were labeled as described above with [^{32}S]methionine and immunoprecipitated with goat anti-MoLV sera (Resources Branch, U.S. National Cancer Institute). Analysis of immunoprecipitated proteins was by SDS–polyacrylamide slab gel electrophoresis.

Results

AbLV-transformed cells express the *abl* oncogene-coded proteins, which are 90–160 kd in size, depending on transformed cell type [50, 52, 58]. The *abl* oncogene product p160 of RAW117 cells can be immunoprecipitated from RAW117 cell lysates by antibodies directed against the shared portion of p160 molecule that is coded by the normal c-*abl* cellular oncogene [59]. Antiserum Ab-T1 contains antibodies directed against p160, and antiserum Ab-T2 contains antibodies directed against p160 determinants plus additional MoLV precursors for the *env* (p80) and *gag* (p65) proteins [52]. Equivalent amounts of the *abl*-encoded p160 is precipitated by Ab-T1 and Ab-T2 from cell lysates of low (RAW117-P) or high (RAW117-H10) metastatic potential cells (figure 1). As a positive control for these experiments, the *abl*-encoded protein p120, synthesized by a different AbLV-transformed cell line, 2M3/M [54], is also immunoprecipitated by these reagents (figure 1).

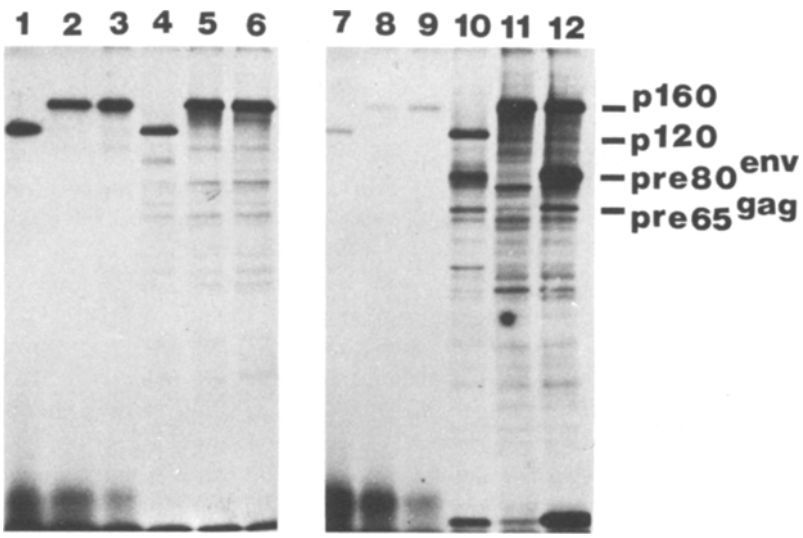

Figure 1. Synthesis and phosphorylation of *abl*-coded p160 in murine large cell lymphoma cells of low (RAW117-P) and high (RAW117-H10) metastatic potential and in AbLV-transformed fibroblasts (2M3/M) by immunoprecipitation with Ab-T1 (lanes 1–6) and Ab-T2 (lanes 6–12). Lanes 1, 4, 7, 10, 2M3/M; lanes 2, 5, 8, 11, RAW117-P; lanes 3, 6, 9, 12, RAW117-H10; lanes 1–3, 7–9, γ-[^{32}P]ATP; lanes 4–6, 10–12, [^{35}S]methionine.

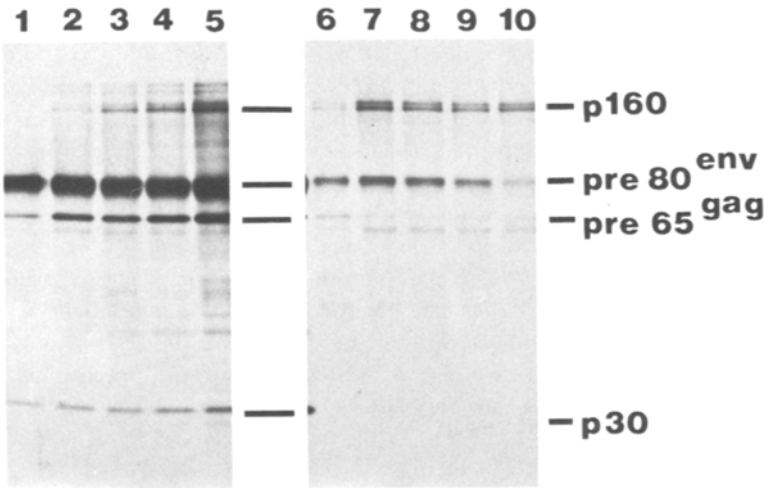

Figure 2. Immunoprecipitation of [^{35}S]methionine-labeled proteins in murine lymphoma cells of low (RAW117-P) and high (RAW117-H10) metastatic potential by goat anti-MoLV. Serial dilutions of goat anti-MoLV were used to precipitate: RAW117-P (lanes 1–5); RAW117-H10 (lanes 6–10).

The amounts of p160 synthesized in low and high metastatic potential RAW117 cells can be compared by pulse-labeling the cells with [^{35}S]methionine and immunoprecipitating p160 from the radiolabeled cell lysates. Similar amounts of [^{35}S]methionine label in p160 are immunoprecipitated with Ab-T1 or Ab-T2 antibodies from RAW117 cell lysates (figure 1; only RAW117-P and RAW117-H10 are shown). Moreover, in pulse chase experiments the p160 from RAW117 cells of low or high metastatic potential exhibit the same half-lives (data not shown).

One of the unique properties of many oncogene-coded proteins is their capacity to be phosphorylated *in vitro* at tyrosine residues [9, 60]. We therefore tested whether p160 would be differentially phosphorylated in the presence of γ-[^{32}P]ATP in low and high metastatic potential RAW117 cells. When γ-[^{32}P]ATP is added to p160 immunoprecipitated by Ab-T1 or Ab-T2 antibodies from cell lysates of RAW117-P or RAW117-H10, and p120 immunoprecipitated from cell lysates of control 2M3/M1 cells, equivalent autophosphorylation of the *abl* oncogene product occurs (figure 1).

To further compare p160 from low and high metastatic RAW117 cells, we labeled cells with [^{35}S]methionine and immunoprecipitated cell lysates with goat anti-MoLV antibodies that bind to the *gag*-encoded region of the p160 molecule [58]. In agreement with the previous experiment using Ab-T1 and Ab-T2 antibodies, excess anti-MoLV antibodies immunoprecipitate similar amounts of p160 from low and high metastatic RAW117 cells (figure 2). Although lower concentrations of the anti-MoLV appear to precipitate more p160 from the high metastasis RAW117-H10 than the low metastasis RAW117-P cells, this discrepancy is probably due to the higher levels of *gag* precursor product and p30 in RAW117-P cells [48]. These latter two components are known to share antigenic determinants with p160 and are recognized by the goat anti-MoLV reagent. Therefore. saturation of these components plus p160 in the RAW117-P cell lysates requires higher antibody concentrations (figure 2).

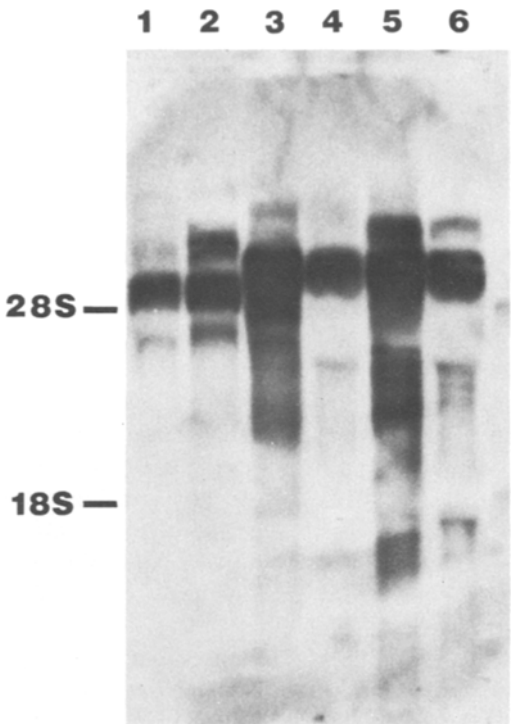

Figure 3. Analysis of *abl*-specific mRNA in murine large cell lymphoma cells of low (RAW117-P) and high (RAW117-H10) metastatic potential and AbLV-transformed fibroblasts. Lane 1, pA^+ RNA of 2M3/M; lane 2, pA^+ RNA of 2M3/M; lane 3, pA^+ RNA of RAW117-H10; lane 4, pA^- RNA of RAW117-H10; lane 5, pA^+ RNA of RAW117-P; lane 6, pA^- RNA of RAW117-P. 18S and 28S ribosomal RNA serve as molecular weight markers.

In addition to the Abelson leukemia virus sequences, RAW117 cells also contain MoLV helper virus. Immunoprecipitation of cell lysates with the goat anti-MoLV antibody will, therefore, yield MoLV-related products and their precursors. Such an experiment reveals that RAW117-P cells synthesize greater amounts of the precursor envelope product (p80) than RAW117-H10 cells (figure 2). This agrees with the observation that RAW117-P cells possess higher levels of gp70 than RAW117-H10 cells [48]. In addition, RAW117-P cells contain more *gag* precursor, detected as a $M_r \sim 65\,000$ product, than RAW117-H10 cells. A mature p30 *gag* product could only be detected in RAW117-P cells (figure 2).

We also compared the expression of specific *abl*-encoded mRNA in RAW117 cells. Polyadenylated and nonadenylated mRNA can be separated on formaldehyde-treated agarose gels, transferred onto nitrocellulose paper, and hybridized with radiolabeled pAbl sub 9 plasmid containing the entire v-*abl* sequence. Both low and high metastatic RAW117 cells express a major species of mRNA of 6·2 kd that hybridizes specifically with the pAbl sub 9 probe, indicating the existence of *abl*-encoded mRNA. Equal amounts of *abl* encoded mRNA are present in low and high metastatic RAW117 cells, suggesting that RAW117 cells of low and high metastatic potential transcribe equally *abl*-specific mRNA (figure 3). Analyses of oncogene expression using probes for the oncogenes *fos* (no expression), *myc* (low expression),

and *myb* (low expression) indicate that these oncogenes are also expressed at similar levels, if expressed, in low and high metastatic potential RAW117 cells (data not shown).

Discussion

We have found that the capacity of AbLV-transformed RAW117 cells to metastasize is unrelated to their expression of a functional *abl* oncogene protein. Although the initial step in cellular transformation in this system may involve switching on the *abl* oncogene, and its expression may be related to the maintenance of transformation, *abl* expression is not required additionally for further steps in malignant progression, such as metastasis. This notion is in agreement with the data of Whitlock *et al.* [57], who showed that the progression of early passages of AbLV-transformed cell clones to more malignant growth behavior does not involve detectable changes in the concentration, phosphorylation or kinase activity of the *abl* oncogene protein. We did not detect differences in the electrophoretic, immunologic or enzymatic properties of *abl*-coded p160 in low and high metastatic potential RAW117 cells; therefore, differences in the amounts of p160 molecules or their activities in cell sublines of widely disparate metastatic phenotypes could not be detected in this system. However, our analyses cannot exclude minor changes in p160 sequence or activity.

Tumors induced by AbLV and cells transformed *in vitro* by AbLV can lose expression of the *abl* oncogene while maintaining their transformed phenotypes [23]. However, here we found that several RAW117 cell sublines and clones all express the *abl* oncogene at equivalent levels. In addition, AbLV-transformed cells can show expression of other oncogenes [28]. Therefore, we examined the expression of several other oncogenes in AbLV-transformed RAW117 cells. If expressed, these oncogenes were also expressed equivalently in low and high metastatic RAW117 cells, suggesting that differential oncogene expression is not involved in regulating RAW117 metastatic phenotype. Similar conclusions were recently reached independently by Kris *et al.* [27] using other murine tumor cell sublines of differing metastatic potentials. They found that B16 melanoma and UV-2237 fibrosarcoma cells express similar amounts of *Ki-ras*, the major oncogene in these cells, and $p21^{ras}$ in both low and high metastatic potential cells.

In RAW117 cells the *abl*-coded product p160 does not vary in expression, but the MoLV coded products, such as gp70, p30, p15 etc. are progressively diminished in their expression with increasing metastatic potential. In highly metastatic RAW117-H10 cells these MoLV-coded proteins are barely detectable [47, 48] and their expression correlates inversely ($r = 0{\cdot}93$) with metastasis [41]. Therefore, the *abl* gene must be controlled differently from the MoLV genes, even though the *abl* gene is inserted into a helper MoLV genome to form the AbLV. In RAW117 cells the loss of cell surface antigenic components [33, 42, 48], decreased sensitivity to macrophage-mediated cytolysis and cytostasis [32, 49] and increased amounts of cell surface receptors for organ recognition [31] are related to high metastatic potential. The products that confer these properties do not appear to be controlled by the transformation-related *abl* oncogene in RAW117 lymphoma cells.

The question of whether oncogene expression at any level is required for the appearance of metastatic cells within a tumor cell population could not be answered by our experiments or those of Kris *et al.* [27]. However, Thorgeirsson *et al.* [55] have used transfection of 3T3 cells with the *ras* oncogene to produce *ras*-expressing

metastatic sarcomas. In contrast, oncogene expression in metastases of some human cancers, such as colorectal carcinomas, is low or non-existant compared to primary tumors from the same patients [1, 21]. It has been proposed that oncogenes may set in motion the generation of cellular phenotypic diversity and heterogeneity by generating changes in expression of regulatory genes or their promoters similar to the generation of diversity found in the multigene families that code for immunoglobulin molecules [36, 37]. Once tumor cells become malignant and undergo diversification, further changes in oncogene expression may not be critical for achieving the metastatic phenotype.

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