Identification and Characterization of Collaborating Oncogenes in Compound Mutant Mice

Anton Berns, Harald Mikkers, Paul Krimpenfort, John Allen, Blanca Scheijen, and Jos Jonkers

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Abstract

We have used proviral tagging in tumor-prone transgenic mice to identify collaborating oncogenes and genes contributing to tumor progression. This has yielded a series of oncogenes that could be assigned to different complementation groups in transformation: the myc, Pim, Bmi1, and Frat1 complementation groups. Frat1 is involved in tumor progression and appears to function in the Wnt signaling pathway. Overexpression of Frat1 confers a growth advantage to transplanted tumor cells in vivo and to cells grown in vitro at high density. Frat1 might exert its activity by impairing the kinase activity of Gsk3β, which is involved in the degradation of β-catenin.

Pim genes appear to act in tumor initiation and show strong synergy with myc in lymphomagenesis. Overexpression of Pim1 can also overcome some of the proliferative defects caused by defective interleukin signaling supporting a role of Pim1 in cell proliferation. We have applied proviral tagging in compound mutant Eμ-myc/Pim1−/−/Pim2−/− mice to identify genes that can complement for the loss of Pim1 and Pim2 and, therefore, are able to synergize with c-myc in lymphomagenesis. A number of new as well as known genes have been found by this “complementation tagging.” The latter included c-kit, Tpl2, and cyclin D2, suggesting that Pim kinases might act upstream of or parallel to these known proto-oncogenes.

Introduction

Retroviral insertional mutagenesis is a powerful approach to identifying genes that can confer a selectable phenotype to cells or an organism. Mechanistically, insertional mutagenesis can cause deregulated expression of genes or disrupt their coding sequence, which could lead to either an altered activity or its inactivation. Because retroviral integration is a relatively random process, it has a reach different in that it leaves a sequence tag at the “site of the crime” and, therefore, permits the swift identification and characterization of the gene involved in conferring the selective advantage to the cell. Because its mutagenesis spectrum is different from chemical mutagenesis, it will not necessarily yield the same genes; i.e., insertions will not cause point mutations or large deletions and, consequently, if such a point mutation is required, retroviral insertional mutagenesis will not yield such a gene. Proviral tagging is a straightforward procedure when tumor growth in vivo is used as the selected phenotype. We will restrict ourselves here to this application (1), although its utility is much broader. Operationally, proviral tagging is performed by infecting newborn mice with MuLV3 or mammary tumor virus. This gives rise to a long-term viremia (2), with reinfection and retroviral insertion events continuing throughout the life of the animal (for a recent overview of retroviral insertional mutagenesis, see Ref. 1). We will confine ourselves to the studies performed with M-MuLV. Viral integrations in infected cells occasionally lead to the activation of proto-oncogenes, which, in turn, can provide a selective growth advantage to the cell, eventually resulting in a tumor in which the set of proviruses present in the original cell is preserved. The pattern of integrations is made visible by Southern analysis. Typically, several to many clonal or near-clonal proviral integrations are seen. When more than one independently induced tumor carries an integration at a particular locus, this is named a CIS. Such an insertion is invariably of oncogenic significance because the possibility of this happening by chance alone is extremely small. The majority of integrations do not occur in CISs and are of no significance for tumorigenesis, having occurred before relevant oncogenic events in the parental cell and been carried along for the ride. Nevertheless, it is not unusual to find MuLV integrations at more than one known CIS in any given tumor.

This strongly implies that the affected genes in these CISs can collaborate in tumorigenesis because otherwise the later insertions would not have been selected for during tumor outgrowth. The ability of an integrated provirus to unleash the transforming potential of cellular genes has led to the identification of a large number of cellular genes that, upon aberrant expression or truncation, mediate a selective advantage to cells (1). Well-known proto-oncogenes that are frequently found activated by proviral insertion in lymphoid tumors are c-myc, N-myc, and Pim1 (1). The reach of insertional mutagenesis has been further extended by proviral tagging in mice bearing oncogenes in their germ line. Infection of these mice will preferentially lead to tumors carrying provirally induced alterations that synergize with the action of the oncogene present as a transgene; e.g., the infection of Eμ-myc transgenic mice resulted in the outgrowth of tumors carrying proviral insertions affecting the collaborating oncogenes Pim1 and Bmi1 or Gfi1 (3, 4) but not myc because, apparently, no selective advantage is associated with the activation of myc in cells that already harbor a highly expressed myc transgene. Applying this approach to a range of mutant mice carrying different oncogenes permits assignment of the various oncogenes to distinct complementation groups in transformation.

Further expansions along this theme take advantage of the possibility of prolonging the life span of primary tumors by transplanting them into syngeneic hosts. This permits the outgrowth of more malignant clones that were arising in the primary tumor as a result of ongoing insertional mutagenesis. Genes frequently targeted later in the tumorigenic process likely contribute to tumor progression and probably belong to the category of genes conferring accelerated growth or metastatic potential to tumor cells. Finally, proviral tagging in compound mutant mice harboring activated proto-oncogenes as well as disrupted synergistic proto-oncogenes can be used to specifically search for genes acting in defined signal transduction pathways. This focused search for genes acting in a specific pathway has been called “complementation tagging.” The latter two approaches will be discussed in more detail.

Materials and Methods

For the generation of transgenic and knockout mice and retroviral insertional mutagenesis, standard procedures were used, as described previously (5). Pim1 and Bmi1 transgenic mice were made using the TDK expression cassette. In this cassette, the immunoglobulin heavy chain enhancer is combined with the Pim1 promoter. The M-MuLV

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3 The abbreviations used are: MuLV, murine leukemia virus; M-MuLV, Moloney MuLV; CIS, common insertion site; wt, wild-type.
long terminal repeat is used for termination of transcription. The lines were made within the FVB/N background. Transgenic Eμ-myc mice that were produced previously (3) were crossed for several generations onto the FVB/N background. The same holds for the Pim1 and Pim2 knockout mice. Fra1 transgenic mice were generated in the FVB/N background. Cloning of new common proviral insertion sites was performed essentially as described. Host DNA flanking the proviral integrations was cloned using a PCR-based method, as described previously (3).

Results

Identification of Collaborating Oncogenes. We have concentrated on transgenic mice overexpressing oncogenes involved in lymphomagenesis as a starting point to identifying new collaborating oncogenes. Earlier studies have shown that retrovirus infection in Eμ-myc transgenic mice resulted in the frequent activation of the Pim1, Pim2, and Bmi1 genes in the B-cell lymphomas, which are the predominant tumor type arising in these mice (3–5). In addition, insertions in the PolII locus, which were frequently found in these tumors, appeared to be associated with the up-regulation of the Gfi1 gene (6). Depending on the transgene present, different sets of oncogenes can be found activated upon insertional mutagenesis of these tumor-prone mice. In this way, complementation groups in transformation can be defined, belonging to the myc, Pim1, or Bmi1 category (see Fig. 1). A number of different genes have been assigned to these three categories, based upon their mutually exclusive activation (Table 1); e.g., in TDK-Pim1 transgenic mice, activation of c-myc, N-myc, and Gfi1 was observed, whereas in Bmi1 transgenic mice, activations of Pim1, Pim2, c-myc, and N-myc were predominant. In a small percentage of the tumors, Gfi1 activations were found, indicating either that Bmi1 and Gfi1 do not fully complement each other or that the expression level of Bmi1 is insufficient to fully obliterate the need for Gfi1 activation. These experiments showed a consistent integration pattern, i.e., mostly the same subset of genes were found, irrespective of whether T- or B-cell lymphomas arose, the only exception being Bmi1, the activation of which appears to be restricted to B-cell lymphomas in the mouse. The expression pattern of the transgene determines to a large extent the type of tumor. Consequently, Eμ-myc transgenic mice, which express the myc oncogene almost exclusively in the B-cell compartment, will primarily yield B lymphomas, whereas H2-K-myc transgenic mice, which express the transgene throughout the hematopoietic system, show a preponderance of T-cell lymphomas. TDK-Bmi1 transgenic mice show predisposition to both B- and T-cell lymphomas.

Identification of Oncogenes Involved in Tumor Progression. Because insertional mutagenesis is a continuous process during tumor development, a situation is created in which sequential activations of genes that can contribute to the various stages of tumorogenesis can take place. One would expect that, in the early phases of the tumorigenic process, (in)activation of genes directly involved in cell proliferation plays a predominant role, whereas later in the disease, genes that might contribute to tumor progression become the target. To address this experimentally, cell suspensions were made of primary lymphomas induced by MuLV infection in TDK-Pim1 or H2-K-myc transgenic mice and transplanted to a number of independent syngeneic hosts (Fig. 2). Tumors that grew out were collected, and the proviral integration pattern was compared with that of the primary tumor by Southern blot analysis. All of the transplanted tumors harbored a number of provirus-specific bands that were also present in the primary tumor. However, the transplanted tumors frequently carried additional bands that were common to the independent outgrown transplants of the same primary tumor but that were not or were hardly detectable in the primary tumor samples. The presence of the same proviral insertion site in independent transplants indicates that this insertion was already present in a small fraction of the primary tumor cells. Apparently, this cell clone, which had a selective growth advantage upon transplantation, was generated long after expansion of the primary tumor had started and, therefore, represented a tumor progression event. A series of these additional insertions were cloned, and we sought to determine whether one of these insertions represented a CIS.

In this way, new loci were identified that were specifically involved in tumor progression. In one of these loci, we identified the relevant

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<tr>
<th>Group</th>
<th>Genes</th>
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<tr>
<td>1</td>
<td>c-myc, N-myc, L-myc</td>
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<tr>
<td>2</td>
<td>Pim1, Pim2, Pim3, ILS, Tpl2, c-kit, cyclin D2</td>
</tr>
<tr>
<td>3</td>
<td>Gfi1, Gfi1b, Bmi1</td>
</tr>
<tr>
<td>4</td>
<td>Fra1</td>
</tr>
<tr>
<td>5</td>
<td>New common insertion sites</td>
</tr>
</tbody>
</table>

Fig. 2. Identification of tumor progression genes by proviral tagging and primary tumor transplantation. Aliquots of cell suspensions of primary tumors were transplanted into syngeneic hosts, and tumors were allowed to grow out. The proviral integration patterns of the primary and transplanted tumors are shown (Southern blot of EcoRI-digested DNA probed with a specific U3 long terminal repeat probe of M-MuLV). Arrowheads, additional proviral insertions in the transplanted tumors. Sizes of HindIII-digested λ DNA fragments are indicated in kbp (left).
gene that conferred this selective advantage to transplanted tumor cells. We have named this gene Frat1 (7). Frat1 encodes a small protein of 274 amino acids. Its role in tumor progression was established by monitoring the growth characteristics of suitable cell lines after retroviral transduction. Tumor cell lines derived from spontaneously arising tumors in TDK-Pim1 transgenic mice in which we transduced the Frat1 gene together with a lacZ marker showed a significant better growth capacity upon transplantation than cells that were transduced with the lacZ marker only. This has been most clearly demonstrated by transplanting mixtures of transduced and nontransduced cells (7). Recently, a homologue of Frat1 was identified as a binder of Gsk3 in Xenopus (8). We have confirmed these observations and shown that the mouse and human Frat1 can act as potent inhibitors of Gsk3 (data not shown).

Pathway Identification by Complementation Tagging. We have applied proviral insertional mutagenesis to identify genes that function in defined pathways. We reasoned that targeted disruption of genes that are frequently activated by proviral tagging might create a situation in which insertional mutagenesis might specifically lead to the activation of genes that can act downstream or in parallel of the inactivated gene product. Because loss of Pim1 and/or Pim2 had no detrimental effect on the viability of mice (9), we generated various compound mutant genotypes and performed an insertional mutagenesis screen to search for genes that would complement the loss of these genes (7). Also, a homologue of Frat1 was duly bred onto a lacZ transgenic mice in X. laevis (8). We have confirmed these observations and shown that mouse and human Frat1 has been shown to act as a potent inhibitor of Gsk3 (data not shown).

Table 2. Features of oncomice/knockout mice

<table>
<thead>
<tr>
<th>Proto-oncogene</th>
<th>Transgene</th>
<th>Transgenic phenotype</th>
<th>Knockout phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pim1</td>
<td>TDK-Pim1</td>
<td>Predisposition</td>
<td>No obvious phenotype</td>
</tr>
<tr>
<td>Pim2</td>
<td>TDK-Pim2</td>
<td>B and T lymphomas</td>
<td>No obvious phenotype</td>
</tr>
<tr>
<td>Pim1 + Pim2</td>
<td>Eμ-myc</td>
<td>B lymphomas</td>
<td>No obvious phenotype</td>
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<tr>
<td></td>
<td>H1-K-myc</td>
<td>B and T lymphomas</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>Bmi1</td>
<td>TDK-Bmi1</td>
<td>B and T lymphomas</td>
<td>Developmental defects</td>
</tr>
<tr>
<td>Gfi1</td>
<td>TDK-Gfi1</td>
<td>B and T lymphomas</td>
<td>Not targeted</td>
</tr>
<tr>
<td>Frat1</td>
<td>TDK-Frat1</td>
<td>No spontaneous tumors</td>
<td>Acceleration in crosses</td>
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*A CML, chronic myelogenous leukemia.*

Discussion

Proviral tagging is an efficient methodology to identify new oncoproteins. In addition, the method permits identification of complementation groups of transformation. Every complementation group consists of genes that can substitute for each other in promoting the function of Pim genes. Therefore, this proviral tagging experiment was extended to the Eμ-myc/Pim1-/-/Pim2-/- background. It should be pointed out that the Pim1/2 double-knockout mice show a phenotype that is similar to that of Pim1 single knockouts (Table 2; Refs. 10 and 12). The deficiencies in responses to growth factors in vitro are somewhat more severe, but this still appears to be of little consequence for the health of the mice or the composition of their hematopoietic compartment. Effects may well be present, but under normal circumstances, they must be subtle.

An Eμ-myc transgene was dually bred onto a Pim1/Pim2 double-knockout background using a breeding program that simultaneously generated myc transgenic mice on wt and Pim1 and Pim2 single-knockout backgrounds as controls.

The median tumor latency in the Pim1/2 double-knockout cohort increased by ~3 weeks, as compared to Eμ-myc mice in a wt Pim1/2 background. This was large enough to suggest that the system had been "stressed," but it was not so great an increase that one would fear that an entirely different pathway was used. In this experiment, a smaller increase in latency was also observed on the Pim1 knockout background. Analysis of a sample of tumors (10 per genotype) by flow cytometry indicated that the compositions of the tumor panels by cell lineage were comparable. The B-lineage tumors had slightly more mature surface phenotypes in the absence of Pim1 and Pim2, fitting a pattern seen in unconnected experiments, which points to the possibility that, without functional Pim1 or Pim2, lymphoid cells have a greater tendency to mature.

Proof that Pim-type pathways were still being used came from an examination of the patterns of collaborating proviral insertions. One predominant gene that was targeted appeared to be Pim3, another member of the Pim family (see Fig. 3). However, Pim3 was never found activated in tumor panels of mice that carried at least one functional Pim1 and/or Pim2 allele. Therefore, Pim3 is a less preferred target for activation. Whether this relates to the fact that insertions near Pim3 occur at a lower frequency or whether Pim3 has a reduced oncogenic activity remains unclear. The other genes now known to be activated in the Pim1/2 knockout tumor panel are: Tpl2, which is also a serine/threonine kinase and a common MuLV insertion site in rats (13); c-kit, the receptor for stem cell factor; and cyclin D2. In none of the controls (Eμ-myc/Eμ-myc; Pim1-/-/Eμ-myc; Pim2-/-) were insertions near any of these genes found.

Tpl2 is the homologue of the human COT oncogene, a COOH-terminal truncated protein (14). The characteristic mode of activation by MuLV in rats is insertion of the provirus between exon 7 and 8, resulting in overexpression of a similar COOH-terminal truncated protein (15). In the Pim1/2 knockout tumors, proviral integrations also invariably occurred in intron 7. As a consequence, the COOH-terminal domain of Tpl2 is deleted, resulting in a protein with constitutive kinase activity toward MEK-1 and SEK-1 (16). Both c-kit and cyclin D2 are known proto-oncogenes. We are currently attempting to obtain biochemical evidence for the involvement of Pim within the pathways in which these genes act.

A number of other CISs were found. However, further analysis of these CISs is required to unequivocally identify the genes that are affected by proviral insertion in these tumors.

Discussion

Proviral tagging is an efficient methodology to identify new oncoproteins. In addition, the method permits identification of complementation groups of transformation. Every complementation group consists of genes that can substitute for each other in promoting biological responses.
tumorigenesis in combination with genes belonging to other complementation groups. This suggests that the proteins encoded by the genes within one complementation group have an effect function in common, although their normal physiological role might show little overlap. It provides us with the opportunity to define what activity or effect of a particular protein is really relevant for tumorigenesis. We have started to search for the common denominator that puts the various genes within the same complementation group. Four of these groups, as defined by insertional mutagenesis, can be discerned at the moment (see Table 1). We still have to learn the biochemical connection between these proteins. Pim1 might exert its function by the inactivation of genes through changing the chromatin structure, permitting, in this fashion, inactivation of, e.g., tumor suppressor gene function. Gfi1, which acts in the same complementation group, contains a dominant transcription repression domain. It is now one of the tasks to determine whether these genes have a common target of gene inactivation.

Pim1 is a member of a complementation group that is rather diverse. The activation of Pim1 and Pim2 through interleukin signaling and their capacity, when overexpressed, to overcome the impairment in thymic cellularity in common γ receptor knockout mice, combined with the complementation in Pim1/2 knockouts by c-kit, Tpr2, and cyclin D2 argues in favor of a role of Pim in the cross-talk between interleukin and growth factor signaling. Pim kinases might potentiate growth factor signaling by increasing the activity of stability of one or more components in the pathway. We are currently trying to determine whether the Pim kinases affect one of the known pathways leading to cell cycle entry.

The function of Frat1 has remained elusive for a long time. However, a COOH-terminal fragment of a Xenopus laevis homolog was recently picked up in a yeast two-hybrid screen with Gsk3β (8). Using intact Frat1 protein as bait, we identified other binders that might provide access to upstream pathways differing from Wnt/Frizzled (17). The COOH-terminal fragment of Frat1 is a potent inhibitor of Gsk3β and leads to β-catenin mediated signaling.7 The intact Frat1 does also act as an inhibitor of Gsk3β, although it is less potent. We expect that, in the near future, the upstream components of Frat1 will be identified. Frat1 knockouts have not provided us with any clues, probably because the Frat1 expression pattern overlaps with expression of a close homologue, Frat3. Analysis of double mutants will be required to enable a better understanding of the physiological function of the Frat proteins.

In conclusion, the various tagging strategies in mice make it possible to attack biochemical problems via a genetic route analogous to the powerful techniques used in invertebrate model organisms. The main difference is that mutagenesis and selection is done in somatic cells. It is practical for oncogenes because their collaboration in tumorigenesis can be exploited to encourage activation of substitutes and because the complementing mutations are self-selecting. With only a little more ingenuity, the approach is potentially applicable to a much wider range of problems, provided that selection of revertant cellular phenotypes and sufficient expansion of the cells for analysis can be arranged. The more knockouts become available and the better the tagging systems used for mutagenesis, the more useful this approach will be for investigating biochemical pathways in the cellular systems of higher organisms. It is to be expected that other, more versatile transposons that give access to a greater variety of (nondividing) cells can be developed that do not require an extracellular phase and also lack some of the other disadvantages of retroviruses. Both retrotransposons (18) and DNA transposons from other organisms (19) hold promise in this respect.

Fig. 3. Complementation tagging in compound mutant mice to search for genes that can compensate for Pim deficiency. Proviral insertional mutagenesis was carried out in the various compound mutant backgrounds. Note the almost 100% frequency of Pim2 activation in the Pim1-deficient background. In the Pim1+/Pim2-deficient background genes were identified (e.g., Pim1, Kit, Tpr2, and cyclin D2) that have not been found activated in Pim1-/2-proficient backgrounds.

References

14. Miyoshi, J., Higashi, T., Mukai, H., Ohuchi, T., and Kakinaga, T. Structure and expression pattern overlaps with expression of a close homologue, Frat1, does also act as an inhibitor of Gsk3β, although it is less potent. We expect that, in the near future, the upstream components of Frat1 will be identified. Frat1 knockouts have not provided us with any clues, probably because the Frat1 expression pattern overlaps with expression of a close homologue, Frat3. Analysis of double mutants will be required to enable a better understanding of the physiological function of the Frat proteins.

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Discussion

Dr. Jerry Adams: I just wondered if you felt that the rescue you saw of the cellularity with Pim transgenes in the T-cell lineage might indicate it has a role in impairing apoptosis.

Dr. Berns: Actually, we looked at that a long time ago and did not find that Pim was involved in apoptosis inhibition; on the contrary, it rather looked that overexpression of Pim increases the apoptotic index. In accordance with this, Pim and Bcl2 are effective collaborators, which argues for a different function of these proteins.

Dr. Phillip Sharp: What did you mean by Bmi might act on tumor suppressors? Can you elaborate a little bit on that?

Dr. Berns: Well, first of all we know from our studies on Hox gene expression in Bmi1 transgenic mice that Bmi1 can suppress Hox gene expression. More recently, the group of Maarten van Lohuizen has observed that Bmi1 knockout embryo fibroblasts show an enhanced expression of p16 whereas overexpression of Bmi1 suppressed p16 levels. Although this is an in vitro system and, therefore, possibly somewhat artificial, it would fit very well with the observed lymphomagenesis in Bmi1 transgenic mice in which p16 and p19Arf could be targets for inactivation. But this all needs substantiation.

Dr. Edison Liu: I have one question, and that is, have you found any strains in which there is resistance to some of the transformation as a prelude to looking at modifier genes?

Dr. Berns: Well, we haven’t done a lot of that but it is clear when you use different strains of mice that you get different tumor spectra. This will not necessarily lead to the activation of different subsets of genes. We have been looking in BALB/c, B1/6, 129, and FVB and did not see much of a difference except that the frequency of activation of distinct proto-oncogenes might slightly differ.

Dr. Joseph Pagano: Moving to the situation in Burkitt’s lymphoma, it occurred to me that it would be interesting to look at the expression of some of these collaborators in human Burkitt’s lymphoma, especially comparing, let’s say, the endemic regions in Africa with the sporadic cases that occur in the United States because we have no explanation for why endemicity of Burkitt’s lymphoma occurs, and c-myc translocation with concomitant overexpression is common to both the American sporadic and African endemic form. Can you give any insight into that?

Dr. Berns: Well, we have looked at subsets of human tumors for PIM expression; however, they do not include Burkitt’s lymphomas. It appeared that, in substantial fraction of poorly defined large B-cell lymphomas, Pim is expressed at a high level. However, we have not found any genetic evidence for the involvement of Pim genes in these tumors. So the overexpression might be due to other mutations related to aberrant growth factor signaling, as both Pim1 and Pim2 respond to a number of interleukins. So for the moment there is no evidence that Pim mutations occur in human tumors. Frankly, this is not a concern for me, as one should look at deregulation of pathways, and Pim might be downstream in such a pathway and, therefore, in fact, a potential target for therapeutic intervention.
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