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Expression of the VRK (vaccinia-related kinase) gene family of p53 regulators in murine hematopoietic development

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Abstract The vaccinia-related kinase (VRK) proteins are a new group of three Ser-Thr kinases in the human kinome. VRK proteins are upstream regulators of several transcription factors. VRK1 phosphorylates p53 in Thr-18 within the region of binding to mdm2 preventing their interaction. The tissue distribution of three genes is still largely unknown. In the present report the expression of these genes was analyzed during murine hematopoietic development. The three genes are expressed in fetal liver and peripheral blood, with higher levels between days 11.5 and 13.5, a time when there is a massive expansion of liver cells, and thereafter their expression falls significantly. VRK genes are expressed, particularly at mid-gestation, in embryo thymus and spleen, but in adult thymus and spleen their levels are very low. VRK2 is expressed at lower levels than VRK1 and VRK3 in the mouse embryo. VRK genes play a role during embryonic development of hematopoiesis.

Key words: VRK1; VRK2; VRK3; p53; Ser-Thr kinase; Mouse development; Liver; Hematopoiesis

1. Introduction

The vaccinia-related kinase (VRK) Ser-Thr protein kinases have been identified in the human kinome as a new family, diverging early from the branch that led to casein kinases [1]. These kinases have homology in their catalytic domain to a gene in *Caenorhabditis elegans* that is embryonic lethal [2], and to genes in *Schizosaccharomyces pombe* that are implicated in DNA damage repair [3]. The family is composed of three members, originally identified by the homology of the catalytic domain to the same region of the vaccinia virus B1R kinase [4], a kinase necessary for viral DNA replication. The human VRK1 protein phosphorylates p53 in Thr-18 [5], and has an affinity that is well adapted to the intracellular concentrations of p53 [6]. Among these kinases, only the subcellular localization for VRK1 is known; this kinase is nuclear during interphase [5,7], and dispersed throughout the cell, but not located on the mitotic spindle or chromatids during mitosis (F.M. Vega and P.A. Lazo, unpublished). The three VRK human kinase genes appear to have a ubiquitous expression in adults, but the levels vary greatly depending on the cell line type [4]. It has been suggested that these kinases are expressed at high levels under conditions of proliferation, such as in regenerating liver or tumor cells [4].

The regulation of p53 plays a major role in the control of the cell cycle, proliferation and apoptosis [8,9]. P53 mediates signals that control DNA integrity [10]. Thus, in case of genetic damage, p53 stops the cell cycle to permit its repair or alternatively, if the DNA damage cannot be repaired, the cell undergoes apoptosis and thus the organism is protected [9,11]. The tumor suppressor protein p53 is a key molecule in the integration of cellular responses to many different types of stimulation, both in normal conditions and under different stress situations [12]. Therefore, the intracellular level of p53 is essential for cell cycle control and induction of apoptosis [13]. The regulation of the p53 tumor suppressor protein is mediated by different types of posttranslational modifications, such as phosphorylation, acetylation, sumoylation and ubiquitination [14,15]. The p53 protein is regulated by several kinases [16,17] that respond to different types of activation [12,18]. Some of the p53 phosphorylations take place in its N-terminus [5,19], in a region that is required for binding to its negative regulator mdm-2/hdm-2 [20], preventing their interaction and thus resulting in an increase in p53 stability. The phosphorylation of Thr-18 is the main regulator of the p53–mdm2 interaction [21,22]. Recently some new kinases that phosphorylate p53 in its mdm-2 binding site have been reported [5,6].

In this report we have analyzed the pattern of expression of the VRK gene family of p53 regulatory kinases during murine development in the post-gastrulation mouse embryo, particularly in the context of the hematopoietic system. The ubiquitous expression, though at very different levels, of these genes suggests that they play a basic role in cell physiology, and their inactivation is likely to be lethal in the embryo.

2. Materials and methods

2.1. Mice and organs

BALB/c mice were maintained under pathogen-free conditions in the animal facilities of the ISCIII. Liver, kidney, muscle, thymus and spleen were dissected from 2-month-old BALB/c mice. Timed pregnancies were determined by the observation of the vaginal plug in the morning after overnight mating (gestation day: 0 days after coitus, dpc). Single-cell suspensions from blood, yolk sac (YS), liver (10.5–17.5 dpc), thymus and spleen (13.5–17.5 dpc) were prepared as previously described [23]. Bone marrow (BM) was flushed out by injection of phosphate-buffered saline/2% fetal calf serum with a 25-gauge needle into the femur of 2-month-old BALB/c mice. Viable cells were counted by trypan blue exclusion.

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2.2. RNA extraction from organs in murine embryos

Total RNA was extracted from different microdissected organs and cell suspensions using the phenol–guanidinium method (Trizol reagent, Invitrogen, Carlsbad, CA, USA), followed by ethanol precipitation. The RNA (0.2 μg) was used for cDNA synthesis with Superscript reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

2.3. PCR amplification of murine VRK1, VRK2 and VRK3

The presence of RNA specific for VRK1, VRK2 and VRK3 was determined by semi-quantitative polymerase chain reaction (PCR). A tenth of the reverse transcription (RT) reaction was used as substrate for PCR amplification. For amplification of murine VRK1 (GenBank AA815837) we used primers mVRK1A (5′-ATGCCCCGTGTAAAAGCAGCTC-3′) and mVRK1B (5′-GAAGTACCTTGGTGTTCCTAAG-3′), which amplify a band of 332 nt. The amplification of the murine VRK2 (GenBank AA914007) was performed with primers mVRK2A (5′GGATTTGGTCTGACTGATTTCAAAGG-3′) and mVRK2B (5′-GGAGTGGCCCCATCCAGGAGGAGTG-3′), which amplify a band of 382 nt. The amplification was performed with an initial cycle at 95°C for 5 min, followed by 25 cycles consisting of three steps (95°C for 40 s, 56°C for 40 s, and 72°C for 50 s), and a final cycle at 72°C for 5 min. For murine VRK3 (GenBank BC010473) the primers used were mVRK3A (5′-TGACGCCTCATGTGTCATCCGTTCC-3′) and mVRK3B (5′-TTGTCCTGGTGAATGCCAAAGCCG-3′), which amplify a band of 559 nt. The conditions were an initial cycle at 92°C for 5 min, 30 cycles (92°C for 40 s, 58°C for 40 s, and 72°C for 40 s) and a final cycle at 72°C for 10 min. The primers and PCR conditions of the β-actin PCR used here to normalize the cDNA content were as described [23]. All the reactions were performed with Taq DNA polymerase (Promega, Madison, WI, USA) with 2.5 mM $MgCl_2$. The PCR products were analyzed in a 2% agarose gel. DNA was transferred to a Zeta-Probe (Bio-Rad, San Diego, CA, USA) membrane and hybridized to the corresponding probe at high stringency under conditions previously described [24]. The gel image was analyzed with the Quantity One software using a Bio-Rad FX Imager. For quantification suboptimal exposures within the linear response range were used.

3. Results

3.1. The murine VRK proteins

Of the three murine VRK proteins, only VRK1 has been partially characterized [7]. The other two were identified by their homology to the corresponding human cDNA. The alignment of the expected three murine proteins with the Clustal W program is shown in Fig. 1. The three VRK proteins have high homology in their Ser-Thr catalytic domain. Both VRK1 and VRK2 proteins have the catalytic domain in their amino-terminus, while in the VRK3 protein it is located at the carboxy-terminus. The Ser-Thr kinase domain, including the ATP binding site and the catalytic site, spans a region of approximately 295 residues (Fig. 1), with an overall homology of 73% between the three proteins, with half of the residues identical, and the other half similar. VRK1 has a nuclear localization signal (NLS) in residues 356–360, consistent with its nuclear localization by subcellular fractionation [7]. VRK3 also has a bipartite NLS sequence at positions 49–66, but its subcellular localization in vivo is not known. The VRK2 protein has a hydrophobic membrane-anchoring region at the end of its carboxy-terminus, spanning residues 432–453, and two isoforms with and without this anchor are present in some cell types (S. Blanco, L. Klimcakova and P.A. Lazo, unpublished). VRK1 and VRK2 diverge in their carboxy-terminal regions and have no homology to any other known domain or protein characteristic. This lack of homology also applies to the amino-terminus of VRK3. These divergent regions are likely to be implicated in the regulation of these Ser-Thr kinases.

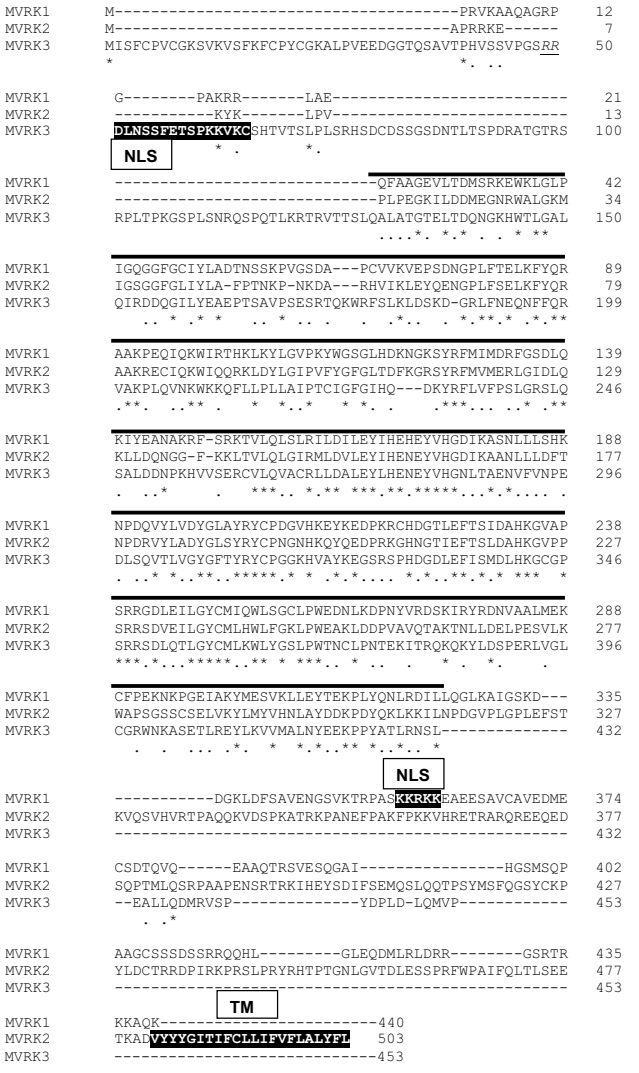

Fig. 1. Alignment of the three murine VRK Ser-Thr kinases. The proteins derived from the cDNA were aligned with the Clustal W program. The conserved Ser-Thr kinase domain is indicated by a bold bar and includes the ATP binding site and the catalytic site. NLS represents the nuclear localization signal in VRK1 and VRK3. TM indicates the transmembrane domain of VRK2. Identical (asterisks) and similar (dots) amino acid residues are indicated. GenBank accession numbers: for murine VRK1 (AA815837), VRK2 (AA914007) and VRK3 (NM_133945).

3.2. VRK gene expression in developing murine liver

In murine post-gastrulation embryonic development the liver undergoes a massive increase in cell number and size, from 10^3 cells to 10^7 total recovered cells per organ [23]. During this time window a simultaneous process of hematopoiesis and liver morphogenesis is taking place in the organ [25,26]. From day 13.5, liver hematopoietic precursors are released and home other hematopoietic niches that become receptive, such as spleen, thymus, and finally by day 17 the bone marrow that becomes the major hematopoietic organ in adulthood [26]. We determined the expression of the three VRK genes in murine embryo liver from days 11.5 to 17.5 (Fig. 2).

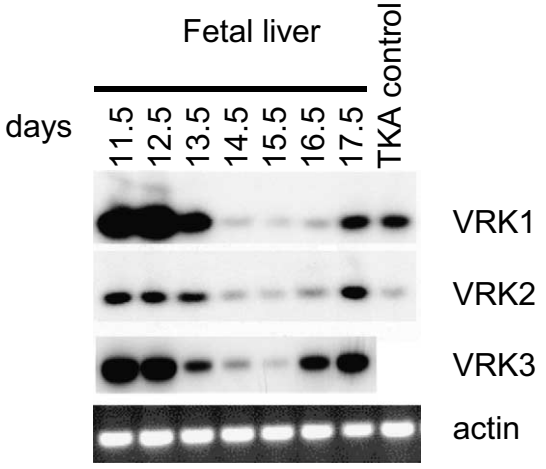

Fig. 2. VRK gene expression in liver embryonic development. The samples comprise from day 11 to 17 that includes the period of massive expansion in liver cells. The exposures shown are longer than those used for quantification. The amount of VRK3 PCR loaded is one tenth of the VRK1 and VRK2. Positive control for VRK3 is not shown. The exposures shown in the figure do not correspond to those used for quantification, which were much shorter.

The three VRK genes are expressed throughout liver development with maximal expression between days 11 and 14, while at day 10.5 only low levels of the transcripts were detected (not shown). The expression is approximately 10- and 20-fold higher for VRK1 and VRK3, respectively, than for VRK2. In both cases, gene expression falls by more than one order of magnitude by day 14.5 (Fig. 2). The relative levels of VRK1 with respect to VRK3 are similar throughout liver development. The levels of VRK2, although reaching a maximum on the same days, between days 11.5 and 13.5, have a smaller overall variation. The expression increases again at day 17.5, and remains positive thereafter (not shown).

3.3. VRK gene expression in hematopoietic embryonic tissues

Peripheral blood was extracted from the embryo umbilical cord and used for VRK expression analysis from days 10.5 to 17.5. The highest levels were detected at days 10.5 and 11.5 and slowly decreased until day 14.5 followed by a drop of at least one order of magnitude in the case of VRK1 at day 15.5. In the case of VRK2 and VRK3 the drops, if any, in level after day 14.5 are significantly smaller and the values are very similar throughout the whole embryonic period (Fig. 3A).

VRK gene expression was also analyzed in thymus and spleen, between days 13.5 and 17.5. There is always a relatively high expression, particularly for VRK1 and VRK3 in these locations. As happened in liver and blood, VRK2 signals are much lower (10-fold). In the two organs there appears to be a stable level of expression for each VRK gene, regardless of their individual expression differences (Fig. 3B).

3.4. Low expression of VRK1 and VRK2 genes in the YS

The extra-embryonic layers of the amnion and YS play an important nutritional role for the post-implantation embryo. The YS also supports primitive and definitive erythromyelopoiesis at early stages after gastrulation (from 7 to 10 dpc) until it is replaced by the liver as the major hematopoietic organ at 11.5 dpc [26]. The YS expresses the three VRK genes between days 10.5 and 15.5. VKR1 and VKR2 are expressed at lower levels than VKR3 (diluted 10-fold in the gel shown) in these samples, and by day 15 there is an important drop in their expression (Fig. 3C).

3.5. VRK gene expression in murine adult organs

The expression of the three murine kinases, VRK1, VRK2 and VRK3, was studied by RT-PCR in tissues from adult mice. As shown in Fig. 4, VRK1 and VRK2 expressed clearly different levels depending on the tissue. Kidney, muscle and liver from 2-month-old BALB/c mice highly express the kinases. The adult hematopoietic tissues analyzed here (thymus, spleen and bone marrow) expressed low levels of VRK1 and VRK2. On the other hand, VRK3 appears to be expressed more homogeneously in all tissues tested, except in spleen where its expression is also decreased. These expression data were confirmed by Northern blot analysis (not shown).

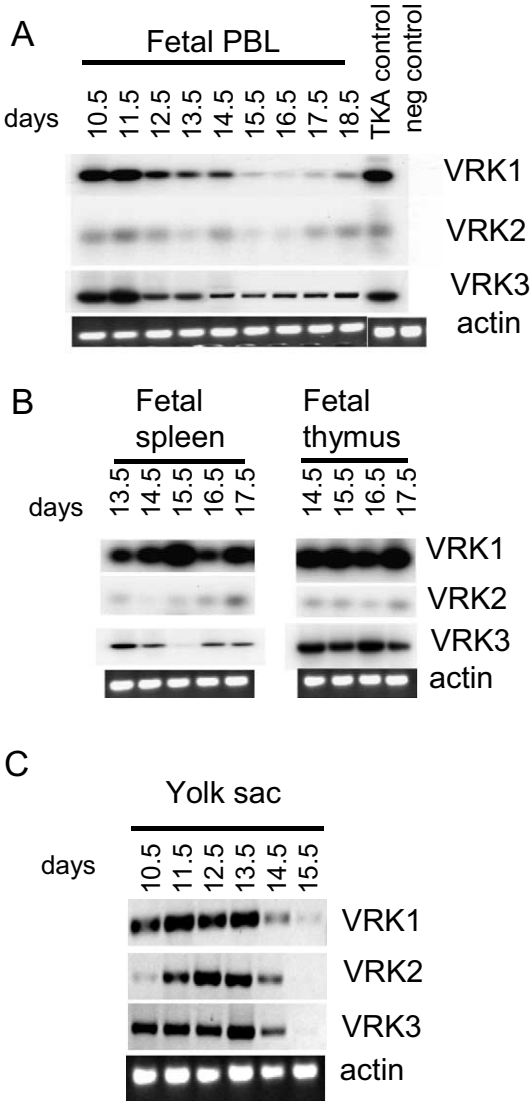

Fig. 3. VRK gene expression in peripheral blood from embryo umbilical cord (A), spleen and thymus (B) and YS (C). The amount of VRK3 PCR loaded in A and B is one tenth of the VRK1 and VRK2. The exposures shown in the figure do not correspond to those used for quantification, which were much shorter.

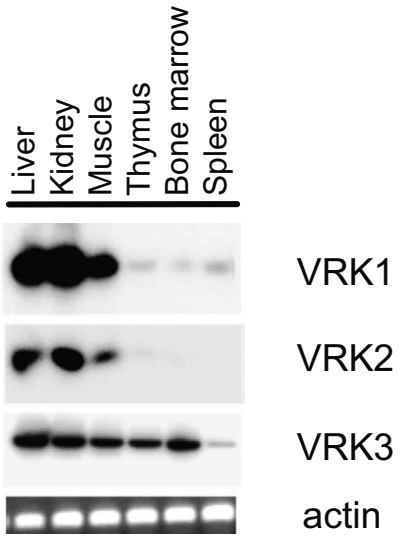

Fig. 4. Expression of VRK genes in adult murine organs. The samples were studied by RT-PCR in a manner similar to embryo samples. Quantification was performed with respect to actin controls, all of them in the linear response range. The exposures shown in the figure do not correspond to those used for quantification, which were much shorter.

4. Discussion

The expression of VRK genes suggests mostly a common pattern of expression for the three genes regarding timing and levels. During murine development the fluctuations in the levels of the three members of the VRK family follow a similar pattern in liver and peripheral blood. In general there is a peak of expression that occurs between days 11.5 and 13.5 followed by a significant drop in the levels, particularly in peripheral blood. In fetal liver the highest level of expression corresponded to the time when the liver undergoes a major expansion in cell number, where the increase corresponds to very short cell cycles, since there are at least 20 cells doubling in 3 days.

The expression of VRK1 in liver development, during the phase of its massive expansion, is consistent with the relatively higher expression detected in regenerating liver and in hepatomas, suggesting that in this cell type it is associated with the increase in cell number [4], or with its early hematopoietic role. However, its high expression in adult liver, where there is no cell proliferation, suggests a more complex role for these proteins in cell biology.

The VRK proteins are members of a new signaling pathway and their targets have not yet been characterized, despite their presence in almost all cell types tested. One of the protein targets of the VRK1 and VRK2 kinases is p53, both specifically phosphorylate p53 in Thr-18 [5]. The consequence is the stabilization and accumulation of p53. This effect is likely to represent a basic mechanism by which p53 is ready to respond to its signals. For the p53 protein to be functional to be able to initiate a response to damage, a minimum level of its activity has to be preserved in the cell at all times. The mechanism that maintains p53 in its readiness state is very little known. The VRK proteins are candidates to be a component of this mechanism, particularly VRK1. Consistent with this role could be a ubiquitous or near-ubiquitous expression and a certain degree of redundancy in their activities.

In adult organs the three genes are widely expressed and the ratio of the three is similar. This suggests that VRK genes are likely to play a basic role in cell biology. Among these functions, and based on the nature of some of their phosphorylation targets, they must play a role related to cellular protection, constituting a system ready for immediate response. Because of this role as a possible part of cell protection mechanisms, they are likely to be candidates for a role as tumor susceptibility or tumor suppressor genes. This is consistent with the very high frequency of loss of heterozygosity (LOH) of markers flanking the VRK1 gene in region 14q32 detected in neuroblastomas [27], nasopharyngeal [28] and colorectal carcinomas [29], and in the blastic crisis of chronic myelogenous leukemia [30]. LOH flanking the VRK2 locus in 2p14 has been reported in lung [31] and adrenocortical [32] cancers.

The information on gene expression of VRK genes is also relevant for the future design of knock-out strategies to determine the individual VRK gene role, since there is a potential risk of redundancy. Furthermore, because of their expression during early phases of liver development, there is a significant risk that knock-out animals will be non-viable, as in *C. elegans* [2], unless they are generated as conditional knock-out mice and the genes are induced after liver development.

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