Generation of Mice With a Conditional Allele of the p120 Ras GTPase-Activating Protein

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Summary: p120 Ras GTPase-activating protein (RasGAP) encoded by the rasa1 gene in mice is a prototypical member of the RasGAP family of proteins involved in negative-regulation of the p21 Ras proto-oncogene. RasGAP has been implicated in signal transduction through a number of cell surface receptors. In humans, inactivating mutations in the coding region of the RASA1 gene cause capillary malformation arteriovenous malformation. In mice, generalized disruption of the rasa1 gene results in early embryonic lethality associated with defective vasculogenesis and increased apoptosis of neuronal cells. The early lethality in this mouse model precludes its use to further study the importance of RasGAP as a regulator of cell function. Therefore, to circumvent this problem, we have generated a conditional rasa1 knockout mouse. In this mouse, an exon that encodes a part of the RasGAP protein essential for catalytic activity has been flanked by loxP recognition sites. With the use of different constitutive and inducible Cre transgenic mouse lines, we show that deletion of this exon from the rasa1 locus results in effective loss of expression of catalytically-active RasGAP from a variety of adult tissues. The conditional rasa1 mouse will be useful for the analysis of the role of RasGAP in mature cell types. genesis 45:762–767, 2007. © 2007 Wiley-Liss, Inc.

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Ras is a small inner membrane-localized GTP-binding protein that controls numerous cell processes including growth, death, differentiation, migration, and polarity (Matozaki et al., 2000; Olson and Marais, 2000). Activating mutations of Ras are observed commonly in human cancer (Downward, 2003). Ras is active in its GTP-bound state and inactive in its GDP bound state that results from Ras hydrolysis of GTP. In its GTP-bound form, Ras triggers the activation of Ras effector molecules such as Raf-1 and phosphatidylinositol 3-kinase (Rodriguez-Viciana et al., 1994; Vojtek et al., 1993). The intrinsic GTPase activity of Ras is low and is considered constant. Instead, other proteins are thought to control the ratio of Ras-GTP to Ras-GDP during cellular signal transduction. One such class of Ras-regulating proteins are guanine nucleotide exchange factors (GEFs) (Bos et al., 2007). Upon juxtaposition to Ras, GEFs eject GDP from the Ras guanine nucleotide-binding pocket. Since the concentration of GTP is higher than that of GDP in the cytosol, the evacuated Ras preferentially binds GTP resulting in Ras activation. Another class of Ras-regulating proteins are Ras GTPase-activating proteins (RasGAPs) (Bos et al., 2007; Donovan et al., 2002). RasGAPs increase the ability of Ras to hydrolyze bound GTP by several orders of magnitude resulting in Ras inactivation. A number of RasGAPs have now been described including neurofibromin, RASA1-4, RASAL1 and L2, and SynGAP (Bernards, 2003; Bos et al., 2007). Amongst these, neurofibromin and p120 RasGAP (RasGAP) are the prototypical members. Like other RasGAPs, RasGAP contains several modular binding domains in addition to its GAP domain. These include two Src homology-2 (SH2) domains, an SH3 domain, and pleckstrin homology (PH) and protein kinase C2 homology domains (see Fig. 1). These domains are thought to mediate recruitment of RasGAP to receptors and membranes whereupon it becomes localized to Ras. As a negative-regulator of Ras, RasGAP has been implicated in signal transduction through numerous different receptors, e.g., the platelet-derived growth factor (PDGF), epidermal growth factor, ephrin, insulin, colony-stimulating factor-1 and T cell antigen receptors (Downward et al., 1990; Holland et al., 1990).
et al., 1997; Kaplan et al., 1990; Margolis et al., 1990; Pronk et al., 1992; Reedijk et al., 1990). In addition, Ras-GAP has been shown to regulate cell death in response to mild genotoxic stress through Ras-independent mechanisms (Yang and Widmann, 2001; Yang et al., 2004).

Recently, inactivating mutations of the human RASA1 gene have been shown to cause a novel clinical disorder named capillary malformation-arteriovenous malformation (Boon et al., 2005; Eerola et al., 2003). Furthermore, a targeted null mutation of rasa1 in mice causes early embryonic lethality through Ras-independent mechanisms (Henkemeyer et al., 1995). These findings suggest an important physiological function for RasGAP at least in endothelial cells and neuronal cells. Studies of fibroblasts derived from rasa1 mutant embryos have additionally revealed a role for RasGAP in directed movement and in PDGF receptor signal transduction in this cell type (Kulkarni et al., 2000; van der Geer et al., 1997). However, because of early embryonic lethality of the rasa1 mutant mice, it has not been possible to use these mice as a model with which to further study the influence of inactivation of RasGAP expression in adult tissues. To this end, we generated a conditional allele of rasa1 in mice.

The rasa1 gene contains 25 exons, of which exon 18 encodes the entire arginine finger loop region of the GAP domain essential for accelerated Ras hydrolysis of GTP (see Fig. 1). Therefore, as a targeting strategy, we attempted to flank exon 18 with loxP sites (see Fig. 2). Following excision of exon 18 by Cre-mediated recombination, the rasa1 transcript would, at the most, be expected to direct the expression of catalytically-inactive RasGAP molecules that lack a functional GAP domain. Furthermore, nonsense sequences would be contained in transcripts from this allele as exon 17 would remain unspliced or spliced to downstream exons all of which are out of frame with exon 17. Theoretically, therefore, transcripts could be rapidly degraded by nonsense-mediated RNA decay, resulting in complete loss of RasGAP expression (Conti and Izaurralde, 2005).

To demonstrate disruption of RasGAP expression, mice harboring a rasa1 floxed allele were crossed with trans-
genic mice that express Cre in different immune cell lineages. Cre transgenic lines examined included Lck-Cre (T cell lineage), Cd19-Cre (B cell lineage) and LysM-Cre (myeloid lineage) (Clausen et al., 1999; Hennet et al., 1995; Rickert et al., 1997). Shown are the results of anti-RasGAP Western blots of whole thymus and spleen cell suspensions from Lck-Cre transgenic mice homozygous for wild-type or rasa1 floxed alleles (Fig. 4a). In thymuses of homozygote floxed mice, expression of full-length RasGAP was almost extinguished. Of the trace amounts of

FIG. 3. Southern blotting and PCR genotyping of targeted and conditional rasa1 alleles. (a) Neomycin-resistant ES cell clones were screened for correct targeting of rasa1 by Southern blotting of genomic DNA. Shown are Southern blots of one several correctly-targeted clones using the indicated restriction enzymes and probes (see Fig. 2). (b) The ES clone shown in (a) was used to generate chimeric mice, which were bred with wild-type C57BL/6 mice to achieve germline transmission of the targeted allele. Shown is a Southern blot of tail DNA from four littermate pups from this cross. Pup 1 has inherited the targeted allele. (c) Mice with a targeted rasa1 allele were crossed with actin-Flp transgenic mice to delete the NeoR cassette. Resulting heterozygote rasa1 floxed mice were intercrossed to generate wild-type, heterozygote and homozygote rasa1 floxed progeny. Genotype was determined by PCR of tail DNA using forward and reverse primers shown in Fig. 2. PCR products of 685 and 545 bp are generated from rasa1 floxed and wild-type alleles, respectively.

FIG. 4. RasGAP protein expression in rasa1 floxed Cre-transgenic mice. (a) RasGAP protein expression in thymus and spleen of Lck-Cre transgenic wild type and homozygote rasa1 floxed mice was determined by Western blotting using a RasGAP antibody that reacts with NH2-terminal SH2 and SH3 domains RasGAP. Blots were reprobed with an actin antibody to verify equal protein loading. Note the almost complete loss of expression of RasGAP in thymus of the rasa1 floxed mice. Note also that no unique lower molecular mass RasGAP antibody-reactive bands are present in rasa1 floxed tracks. Identical results were obtained with an antibody that is specific for the RasGAP SH3 domain (not shown). (b) Homozygote rasa1 floxed mice with or without an inducible ubiquitin-Ert2-Cre transgene were injected with tamoxifen intraperitoneally on two consecutive days. Seven days later mice were sacrificed and expression of RasGAP in different tissues was determined by Western blotting as in (a). Equivalent protein loading was demonstrated by reprobing of blots with a GAPDH antibody.
protein that remained, this could be accounted for by the presence in thymus of nonT lineage cells such as macrophages, dendritic cells and epithelial cells that would not be expected to express Cre (~2–5% of total thymic cells). Less RasGAP deletion was noted in whole spleen (Fig. 4a). However, this is consistent with the fact that T cells comprise only 20–30% of total splenocytes. In lymph node (LN), for example, where T cells comprise 60–70% of total cells, levels of RasGAP expression are reduced correspondingly, consistent with almost complete loss of RasGAP expression in the T cell lineage (not shown).

Similar to results obtained with Lck-Cre, homozygous rasa1 floxed mice with Cd19-Cre or LysM-Cre transgenes express much reduced levels of full-length RasGAP protein in splenic B cells and in bone-derived macrophages, respectively (not shown). Experiments aimed at understanding the consequences of RasGAP loss in different immune cell lineages are ongoing. Preliminary evidence indicates that RasGAP is dispensable for the development of immune cell lineages although performs an important functional role in mature cells.

Mice with a rasa1 floxed allele were also crossed with mice that carry an inducible Cre-ERT2 transgene under the control of a ubiquitin promoter (Ruzankina et al., 2007). The ubiquitously-expressed Cre-ERT2 protein product encoded by this transgene is normally retained within the cytoplasm. However, upon administration of tamoxifen to mice, Cre-ERT2 is translocated to the nucleus whereupon it has access to nuclear DNA and is able to cut and splice loxP sites. Homozygous rasa1 floxed mice with or without the Cre-ERT2 transgene were thus given single injections of tamoxifen on two consecutive days. After 1 week, mice were then sacrificed and expression of RasGAP in different tissues was assessed by Western blotting as before (Fig. 4b). As shown, substantial deletion of RasGAP was observed in each of thymus, spleen, LN and kidney of rasa1 floxed Cre-ERT2 transgenic mice following tamoxifen treatment. Slightly less deletion was observed in lung and brain. However, in subsequent experiments we determined that, when given two repeat tamoxifen injections on consecutive days 1 week after the initial injections, RasGAP expression was almost completely lost in these tissues (as determined in Western blotting experiments performed 1 week later - not shown). Therefore, these mice will be useful for study of the influence of induced loss of RasGAP expression in a variety of tissues of adult mice. Up to 3 months after tamoxifen treatment, mice remain healthy and show no obvious morphological signs of disease. However, detailed analyses of cell function have yet to be performed.

Importantly, in all tissues to which Cre has been targeted, lower molecular mass forms of RasGAP have not been identified in Western blots (Fig. 4a). Absence of these forms cannot be explained by simple loss of antibody epitopes from truncated RasGAP protein(s) since antibodies used in Western blot experiments were generated against NH2-terminal SH2 and SH3 domains. Thus, deletion of exon 18 appears to result in a null rasa1 allele with complete loss of RasGAP expression.

To examine if transcripts generated from the rasa1 exon 18-deleted allele were of low abundance relative to transcripts generated from the wild-type rasa1 allele, we performed RT-PCR analyses upon thymus RNA from heterozygous rasa1 floxed mice with an Lck-Cre transgene (see Fig. 5). Based upon analysis of protein expression in homozygous rasa1 floxed Lck-Cre transgenic mice (Fig. 4a), almost 100% deletion of the floxed allele is expected in the heterozygotes. Using a forward primer located in exon 17 and reverse primers located in exons 19, 21, or 23, it was demonstrated that transcripts generated from the mutant allele are expressed much less abundantly than transcripts generated from the wild-type allele in the same tissue (see Fig. 5). Furthermore, these analyses indicate that in mutant transcripts, exon 17 is spliced exclusively to exon 19 and not to other downstream exons. Results of RT-PCR reactions using a forward primer based on exon 13 with the same reverse primers concurred with these conclusions (not shown). Low abundance of mutant transcripts is probably explained by nonsense-mediated RNA decay and is likely a major contributing factor to our inability to detect truncated lower molecular mass RasGAP forms in Western blots. Additionally, any lower molecular forms that are produced contributing factor to our inability to detect truncated lower molecular mass RasGAP forms in Western blots. Additionally, any lower molecular forms that are produced may be subject to rapid proteolytic degradation.

METHODS

Generation of Conditional rasa1 Knockout Mice

A rasa1 targeting construct was assembled in p-loxP-2FRT-PGKneo (Charles et al., 2006). 5´ and 3´ arms and

FIG. 5. Relative abundance of wild-type rasa1 and rasa1 exon 18-deleted transcripts in thymus. Total RNA from thymus of Lck-Cre transgenic heterozygote rasa1 floxed mice was analyzed by RT-PCR using a forward primer based in exon 17 and reverse primers based in exons 19, 21, or 23. PCR products generated from wild-type and mutant rasa1 transcripts are indicated. For each primer pair, the size of the smaller PCR product is consistent with an exon 17 to 19 splice of the mutant transcript. Note that in each case, the abundance of mutant PCR product is low compared with that of the wild-type PCR product.
an inter-loxP region were generated by PCR from a C57BL/6 genomic rasa1 BAC clone and were inserted into the EcoRI/KpnI, KpnII/Sall and XhoI sites of p-loxP2FRTPGKneo, respectively. Primers used for generation of PCR products were: 5’armF-5’-GCC CGA ATT CGC GGC CGC TTA GTC TTT CAG GTA TAT AGC-3’, 5’armR-5’-GCC CGG TCA AGT CTG ATT TAC CAG GAG TGA C-3’, inter-loxPR-5’-GCC CGG TCA CAG ATC TAT AAC TTC GTA TAA TGT ATG CAA GAA GTT ATA AAT ATT TGA GGC TAT GAG GAC CA-3’, inter-loxPR-5’-GCC CGT CGA CCA TAT CCA ACT TCA CAT GTG TG-3’, 3’armF-5’-GCC CCT CGA GGA ATT TCC CAC ATG CAT GAT GCC A-3’, 3’armR-5’-GCC CCT CGA GAT ATG TTG TCA TGT AAT TTA-3’. The resulting targeting construct contained a 5’ loxP site inserted in intron 17, a 3’ loxP site and FRT-flankedNeoR cassette inserted in intron 18 (≈900 bp downstream from the 3’ loxP site) and 3.5 kbp of upstream and 2.7 kbp of downstream flanking sequence (see Fig. 2).

Absence of PCR-induced errors was verified by DNA sequencing. The construct was linearized with NotI and electroporated into W4 ES cells of 129/Sv origin. Neomycin-resistant clones were analyzed by Southern blotting for correct targeting. 5’ and 3’ probes used in Southern blotting experiments were generated by PCR from the genomic rasa1 BAC clone using the following primers: 5’probeF-5’-GCA AAA TTT CAT CTA GGA-3’, 5’probeR-5’-CCT CAA TGA ACA AAC TTT-3’, 5’probeF-5’-GAA GTT TAC TTA CAA TTA-3’, 3’probeR-5’-GAT TCT TAT GTC GTC-3’. Of several identified euploid correctly-targeted clones, two were injected into C57BL/6 X (C57BL/6 X DBA/2) blastocysts to generate chimeric mice, which were then bred with C57BL/6J mice to achieve germline transmission of the targeted allele. As determined by Southern blotting of tail DNA, germline transmission was achieved for both clones. Mice that had inherited the targeted allele from one of the ES cell clones (clone no.5041) were bred with C57BL/6 actin-promoter-driven Fbp transgenic mice to delete the NeoR cassette (Rodriguez et al., 2000). Mice were then intercrossed to generate wild-type, heterozygote or homozygote rasa1 floxed mice (see Fig. 3).

Genotyping for rasa1 alleles was performed by PCR of tail DNA using the following primers: F-5’-CAG TTT GTC CAT CTA GCT CGT TTG-3’, R-5’-GAA GTT TGA CTT TGG TTG CTG-3’. Alternatively, rasa1 floxed mice were crossed with C57BL/6 mice carrying a Cre transgene driven by the proximal Lck promoter (Taconic) or an inducible Cre-ERT2 transgene driven by a ubiquitin promoter (Hennet et al., 1995; Ruzankina et al., 2007). Heterozygote rasa1 floxed Cre-positive progeny from these crosses were then backcrossed with heterozygote rasa1 floxed mice to generate wild-type or homozygote rasa1 floxed mice either not expressing or expressing Cre transgenes (see Fig. 4). All experiments performed upon mice were in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Tamoxifen Administration

Two-month-old homozygous rasa1 floxed mice with and without Cre-ERT2 transgenes were administered tamoxifen (MP biochemicals) to induce nuclear translocation of the Cre-ERT2 protein. On the basis of an earlier study, in which the same Cre-ERT2 transgene was utilized, a dose of 0.2 mg/g body weight of tamoxifen was chosen (Ruzankina et al., 2007). Tamoxifen was injected intraperitoneally in 200 μl of autoclaved corn oil (Sigma). Mice were given two injections on consecutive days. Seven days later mice were sacrificed for analysis.

Western Blotting

Lysates of different organs of 2-month-old Lck-Cre transgenic wild-type and homozygous rasa1 floxed mice and from tamoxifen-treated rasa1 floxed mice with and without ubiquitin-Cre-ERT2 transgenes were prepared by dounce homogenization in 1% NP-40 lysis buffer. Expression of RasGAP in lysates was determined by Western blotting using an antibody generated against the SH2 and SH3 domains of RasGAP (Millipore). Blots were stripped and reprobed with actin or GAPDH antibodies to demonstrate equivalent protein loading.

RT-PCR

Total RNA was prepared from thymus of Lck-Cre transgenic heterozygote rasa1 floxed mice with Trizol reagent (Gibco) and reverse-transcribed using a Superscript II kit (Invitrogen) and random primers. cDNA was amplified by PCR using a forward primer based on exon 17 and reverse primers based in exons 19, 21, and 23. Primer sequences are as follows: 17F-5’-CGA GAA ATT CGC CCC-3’, 3’-GGA AGT ATT TCT GAA-3’ and 21R-5’-GTA CGT AAT TCA ACC TCC-3’, 23R-5’-CCC AAG TTC ATC TTA AAA CAT-3’.

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LITERATURE CITED


