Murine ORAI2 Splice Variants Form Functional Ca\textsuperscript{2+} Release-activated Ca\textsuperscript{2+} (CRAC) Channels*\textsuperscript{a}

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The stimulation of membrane receptors coupled to the phospholipase C pathway leads to activation of the Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels. Recent evidence indicates that ORAI1 is an essential pore subunit of CRAC channels. STIM1 is additionally required for CRAC channel activation. The present study focuses on the genomic organization, tissue expression pattern, and functional properties of the murine ORAI2. Additionally, we report the cloning of the murine ORAI1, ORAI3, and STIM1. Two chromosomal loci were identified for the murine orai2 gene, one containing an intronless gene and a second locus that gives rise to the splice variants ORAI2 long (ORAI2L) and ORAI2 short (ORAI2S). Northern blots revealed a prominent expression of the ORAI2 variants in the brain, lung, spleen, and intestine, while ORAI1, ORAI3, and STIM1 appeared to be near ubiquitously expressed in mice tissues. Specific antibodies detected ORAI2 in RBL 2H3 but not in HEK 293 cells, whereas both cell lines appeared to express ORAI1 and STIM1 proteins. Co-expression experiments with STIM1 and either ORAI1 or ORAI2 variants showed that ORAI2L and ORAI2S enhanced substantially CRAC current densities in HEK 293 but were ineffective in RBL 2H3 cells, whereas ORAI1 strongly amplified CRAC currents in both cell lines. Thus, the capability of ORAI2 variants to form CRAC channels depends strongly on the cell background. Additionally, CRAC channels formed by ORAI2S were strongly sensitive to inactivation by internal Ca\textsuperscript{2+}. When co-expressed with STIM1 and ORAI1, ORAI2S apparently plays a negative dominant role in the formation of CRAC channels.

Stimulation of membrane receptors that are coupled to the phospholipase C signaling pathway induces Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER)\textsuperscript{a} and the so-called capacitative Ca\textsuperscript{2+} entry from the extracellular space. The Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels that support the Ca\textsuperscript{2+} entry have been characterized and extensively studied in cells of the immune system but their molecular identity remained elusive until it was reported that CRAC currents are nearly completely absent in T cells of patients, who suffered from a hereditary severe combined immunodeficiency (1). Using linkage analysis, the genetic defect in these patients has been identified as a single missense mutation in the orai1 gene, indicating that ORAI1 is an essential component or regulator of CRAC channel complexes (2). Genome wide RNA interference screens came independently to the same result (2–4). ORAI1, also named CRACM1, is a plasma membrane protein that forms four potential transmembrane segments (2–4). Site-directed mutagenesis of acidic residues in its transmembrane segments 1 and 3 indicated that ORAI1 is an essential pore subunit of CRAC channel complexes (5–7). However, formation of functional channels requires apparently the presence of other proteins besides ORAI1. As shown by recent overexpression studies with ORAI1 and STIM1, ORAI1 has minute effects on endogenous CRAC currents of transfected cells, while STIM1 transfected alone or in combination with ORAI strongly amplifies the endogenous CRAC currents indicating that STIM1 is critically involved in the activation of ionic channels formed by ORAI1 (8–10). The STIM1 protein contains only one transmembrane segment and, interestingly, a single unpaired EF hand Ca\textsuperscript{2+}-binding motif located in the N terminus (11). The C terminus of STIM1 is sufficient to activate CRAC channels (12). Furthermore, STIM1 can be located both in the membrane of the ER and in the plasma membrane and function as ER Ca\textsuperscript{2+} sensor and as cell surface signaling protein, respectively (13, 14). Upon stimulation of the phospholipase C signaling pathway, STIM1 and ORAI1 likely cluster in the ER and plasma membrane, respectively, and thereby form functional ionic channel complexes that support CRAC currents and capacitative Ca\textsuperscript{2+} entry (15).

Besides ORAI1, two additional ORAI isoforms (ORAI2 and ORAI3) have been reported so far (2–4, 8, 16). All three ORAI isoforms are widely expressed in human tissues (2, 16), but interestingly, ORAI2 appears to be the only ORAI isoform that has multiple transcripts (16). Although less effective than ORAI1, ORAI2 also amplifies substantially endogenous CRAC currents and enhances store-operated Ca\textsuperscript{2+} influx when co-expressed with STIM1 in HEK 293 cells (8). By contrast to

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**Murine ORAI2 Isoforms**

ORAI1, however, the overexpression of ORAI2 and STIM1 has almost no effect on the store-operated Ca\(^{2+}\) influx in T cells and fibroblasts from severe combined immunodeficiency patients (16), suggesting that ORAI1 and ORAI2 might differ functionally. In this study, we have analyzed the genomic organization, tissue expression pattern and functional properties of the murine ORAI2. For the purpose of comparison, we have also cloned the murine ORAI1, ORAI3, and STIM1. Two orai2 gene loci and two splice variants of the murine ORAI2 (ORAI2L and ORAI2S) were identified in the present study. By contrast to ORAI1 and ORAI3, ORAI2 appeared not to be ubiquitously expressed in mice tissues. We studied the functional properties of the murine ORAI1 and ORAI2 variants in two cell lines, HEK 293 and RBL 2H3 cells, which are commonly used in studies of overexpressed ORAI1 and endogenous CRAC currents, respectively. Using specific antibodies, we found that these cell lines provide different ORAI and STIM1 backgrounds for the functional analysis of recombinant ORAI proteins. In co-expression experiments with STIM1, we found that the difference between CRAC channels formed by the murine ORAI1 and ORAI2 variants resides in their sensitivity to inactivation by internal Ca\(^{2+}\) and in their levels of functional expression, which appears to be cell background-dependent. Additionally, we detected functional interactions between ORAI1 and ORAI2 variants.

**EXPERIMENTAL PROCEDURES**

**Cloning of orai1, orai2l, orai2s, orai3, and stim1**—The sequences of orai1, orai2l, orai2s, and stim1 were predicted using the GeneFinder software (Deutsches Krebsforschungszentrum, Heidelberg, Germany). We identified several orai2 related EST clones, which indicated that two possible start methionines and a common stop codon are present in the orai2 genes. Therefore, we used two different sets of primers to amplify the cDNAs of orai2 from brain total poly(A\(^{+}\)) RNA by reverse transcription-PCR. The 792-bp cDNA fragment encoding ORAI2L was amplified with the primers 5’-ccgccgccacatgagaagaagagaacac-3’ and 5’-tcacacacgctcgcct-3’, and the 750-bp cDNA fragment encoding ORAI2S with the primers 5’-ccgccgccaccatgcatccggagcct-3’ and 5’-tcacaccacctgcaggctc-3’. The latter primers were also used to amplify orai2 from genomic DNA (annealing conditions: 58 °C, 10 s; elongation conditions: 72 °C, 50 s; 53 cycles). The following primers were used to amplify orai1, orai3, and stim1 cDNAs from mouse brain poly(A\(^{+}\)) RNA (orai1 and stim1) and mouse placenta poly(A\(^{+}\)) RNA (orai3): 5’-ccgccgccacatgtagctcgcctgccc-3’ and 5’-ttgagcttagttggtgccc-3’, 5’-ccgccgccacatgagaagaagagaacac-3’ and 5’-ccgccgccacatgagaagaagagaacac-3’. The antisera were affinity-purified and used at 1:1000 to 1:2000 dilutions. The antibody for human, mouse and rat STIM1 was obtained from BD Biosciences. Protein lysates were prepared from equal numbers of non-transfected and transfected cells. Microsomal membrane protein fractions were prepared from non-transfected HEK 293, RBL-2H3, and Jurkat T cells. Protein concentration was determined using the BCA assay, and 100–150 μg of protein were separated by standard SDS-PAGE or by the method described by Schägger and von Jagow (20) using Tricine as the trailing ion. The latter method allows a superior solution of proteins, especially in the range between 5 and 35 kDa as is the case for ORAI1 and ORAI2, which are identical in the ORAI1 and ORAI2 amino acid sequences from humans, rat, and mouse. The antisera were affinity-purified and used at 1:1000 to 1:2000 dilutions. The antibody for human, mouse and rat STIM1 was obtained from BD Biosciences. Protein lysates were prepared from equal numbers of non-transfected and transfected cells. Microsomal membrane protein fractions were prepared from non-transfected HEK 293, RBL-2H3, and Jurkat T cells. Protein concentration was determined using the BCA assay, and 100–150 μg of protein were separated by standard SDS-PAGE or by the method described by Schägger and von Jagow (20) using Tricine as the trailing ion. The latter method allows a superior solution of proteins, especially in the range between 5 and 35 kDa as is the case for ORAI1 and ORAI2, which are identical in the ORAI1 and ORAI2 amino acid sequences from humans, rat, and mouse.

**Cell Culture and Transfection**—HEK 293 cells and RBL cells expressing the human muscarinic receptor M1 (RBL 2H3) were cultured as previously described (19). Plasmids containing the cDNAs of the murine STIM1, ORAI1, ORAI2L, ORAI2S, GAMMA1, and GAMMA2 were transfected into these cells either individually or in combination using the PolyFect transfection reagent (Quiagen). In the individual transfections, 3 μg of each plasmid were used per cell dish. For co-transfections, the STIM1 plasmid was mixed with one of the plasmids containing ORAI1, ORAI2L, ORAI2S, GAMMA1, and GAMMA2 at a ratio of 1:2. The total amount of plasmid mix was 3 μg per cell dish. In the triple co-transfections, plasmids containing STIM1, ORAI1, and either ORAI2L or ORAI2S were mixed at a ratio of 1:2:1. Accordingly, the total amount of plasmid mix was 4 μg per cell dish. The bicistronic expression vector pdi contained the cDNA of the enhanced green fluorescence protein (GFP) as expression marker. Patch clamp experiments were performed on GFP expressing cells 2–3 days after transfection.

**Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—Polyclonal antibodies recognizing mouse, rat, and human ORAI1 (antibody 1003) and ORAI2 proteins (ab 1004) were generated in-house by immunizing rabbits with epitopes corresponding to the C-terminal amino acid sequences derived from ORAI1 and ORAI2, which are identical in the ORAI1 and ORAI2 amino acid sequences from humans, rat, and mouse. The antisera were affinity-purified and used at 1:1000 to 1:2000 dilutions. The antibody for human, mouse and rat STIM1 was obtained from BD Biosciences. Protein lysates were prepared from equal numbers of non-transfected and transfected cells. Microsomal membrane protein fractions were prepared from non-transfected HEK 293, RBL-2H3, and Jurkat T cells. Protein concentration was determined using the BCA assay, and 100–150 μg of protein were separated by standard SDS-PAGE or by the method described by Schägger and von Jagow (20) using Tricine as the trailing ion. The latter method allows a superior solution of proteins, especially in the range between 5 and 35 kDa as is the case for ORAI1 and ORAI2, which are identical in the ORAI1 and ORAI2 amino acid sequences from humans, rat, and mouse.

**Electrophysiology**—Patch clamp experiments were performed in the tight-seal whole-cell configuration at room temperature using an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany). Ionic currents were recorded during voltage clamp ramps from +80 to −100 mV (duration 150 ms) delivered every 2 s and for at least 100 s. In those experiments, in which the bath solution was exchanged, the ramps were delivered every 0.5 s. The holding potential was 0 mV and the sampling rate was 20 kHz. Capacitive currents were determined and corrected in advance of each voltage clamp ramp. The development of ionic currents over time was monitored using current amplitudes measured at −90 mV in each individual voltage clamp ramp. The current densities at −90 mV were calculated as the current amplitude subtracted by the individual background current and divided by the appropriate initial C\(_{\text{slow}}\) value. The standard pipette solution contained 120 mM CsCl, 8...
mm NaCl, 3 mm MgCl$_2$, 20 µM IP$_3$, 10 mm EGTA and 10 mm Hepes, pH 7.2 (CsOH). Additionally, we used a pipette solution that contained: 120 mm cesium glutamate, 8 mm NaCl, 10 mm BAPTA, 3 mm MgCl$_2$, 20 µM IP$_3$, and 10 Hepes, pH 7.2 (CsOH). The standard bath solution was as follows: 120 mm NaCl, 10 mm CsCl, 2 mm MgCl$_2$, 5 mm CaCl$_2$, 10 mm Hepes, 10 mm glucose, pH 7.2 (NaOH). In some experiments, the Ca$^{2+}$ concentration of the latter solution was increased to 10 mm. For the nominally Ca$^{2+}$-free (NCaF) solution, CaCl$_2$ was removed and for the divalent-free (DVF) solution, CaCl$_2$ and MgCl$_2$ were removed and 10 mM EDTA was added. The recordings obtained with the cesium glutamate pipette solution were corrected to account for a ~10 mV liquid junction potential. The osmolarity of external and internal solutions was 290–310 mosm. Chemicals were purchased from Sigma-Aldrich and Merck; IP$_3$ was obtained from Calbiochem. Data are given as mean ± S.E.

RESULTS

Genomic Organization of the Murine orai2 Gene—The human ORAI1 sequence (2) was used to identify several ortholog EST clones from mice. These EST clones were in turn used to identify orai2 exons within the murine genome. orai2 sequences were found on chromosome SG2 and chromosome 16C1. The comparison of EST clones with the genomic locus on chromosome 5 indicated that at least three different transcripts from the orai2 locus arise by alternative splice events (Fig. 1A). Transcripts 1, 2, and 3 represent the murine EST-clones gi16497752, gi27144817, and gi27135608, respectively. Based on this comparison, we also predicted that the orai2 locus of the chromosome 5 consists of at least 5 exons. The start methionine of the 264-amino acid ORAI2 protein was localized in exon 3. Alternative splicing of transcripts derived from the chromosome 5 locus involves the exons 2 and 3. Accordingly, skipping of exon 3 might yield the expression of a N-terminal-truncated version of 250 amino acids, here referred as ORAI2 short (ORAI2S) to distinguish it from the 264-amino acid full-length ORAI2 (ORAI2 long or ORAI2L). By reverse transcription-PCR, the transcripts encoding both ORAI2L and ORAI2S were amplified from murine brain poly(A)$^+$ RNA.

In addition to the locus on chromosome 5, a second orai2 locus was identified on chromosome 16 (Fig. 1A). The locus on chromosome 16 is intronless and resembles a fusion of exons 1, 4, and 5 of the orai2 locus on chromosome 5. To prove the existence of both loci, we hybridized an exon 5 specific probe to genomic DNA which was restricted by EcoRI. As expected from the restriction analysis of both orai2 loci, two EcoRI fragments of 2.7 and 3.1 kb were detected by Southern analysis (Fig. 1B). Next, we asked whether the orai2 locus of chromosome 16 indeed contains no introns. To answer this question, we used genomic DNA as template and the primers derived from the orai2 exons 4 and 5 for PCR. The exons 4 and 5 of the orai2 locus on chromosome 5 are separated by a 10,536-bp intron (Fig. 1C), and it is therefore unlikely to amplify this DNA under the PCR conditions employed, whereas amplification of a 758-bp fragment is to be expected from the intronless genomic sequence. In fact, this 758-bp fragment was amplified (Fig. 1C), and the sequence of this amplicon differed from the corresponding part of the orai2 locus on chromosome 5 by 4 base
pair exchanges (supplemental Fig. 1). Expression of orai2 on chromosome 16 might lead to a protein related to ORAI2S with a single amino acid exchange at position 130 (N130Y). To test whether both orai2 loci are transcriptionally active, we cloned 10 individual ORAI2S related cDNAs from reversed transcribed poly(A)⁺ RNA from murine brain. Sequence comparison of these clones with the murine genome revealed that they arise from chromosome 5 and not from chromosome 16. Furthermore, we analyzed orai2-related EST clones from murine databases and found that they resembled the sequence of the orai2 locus on chromosome 5, indicating that the orai2 locus on chromosome 16 is silent or only expressed to a low extent. Therefore, we suggest that, in mice, orai2 transcripts are primarily derived from chromosome 5. Interestingly, we found two orai2 loci in the rat genome, on chromosome 12 and chromosome 4, whereas in humans, only one orai2 locus is known on chromosome 7 (2). Additionally, we found single copy genes of the closely related orai1 and orai3 on the murine chromosomes 5G1 and 7F2–7F3, respectively.

Expression of Murine orais and stim1—To compare the tissue expression patterns of orai1, orai2, orai3, and stim1, the full-length cDNAs were cloned from murine tissues and used to probe Northern blots containing poly(A)⁺ RNA of murine tissues and primary cells (Fig. 2). orai1 and stim1 transcripts seemed to be ubiquitously expressed. orai3 transcripts seem to be expressed in the same tissues as orai1 with the exceptions of skeletal muscle and placenta. By contrast, orai2 transcripts are predominantly expressed in brain and to a lower extent in the lung, spleen, and small intestine.

Overexpression of Murine ORAI and STIM Proteins—To determine the expression levels of endogenous ORAI and STIM proteins in cell lines usually used for overexpression experiments, we first analyzed the expression of ORAI1 and ORAI2 variants and STIM1 in HEK 293, RBL 2H3, and Jurkat cells using Northern and Western blots (Fig. 3). Transcripts encoding ORAI1 and ORAI2 were readily detected in RBL 2H3 cells and to lesser extent in HEK 293 and Jurkat cells (Fig. 3, A and D). Similarly, the polyclonal antibody detected ORAI2 in RBL 2H3 cells and to a lesser extent in Jurkat cells but not in HEK 293 cells (Fig. 3, E and F). The ORAI1 expression was detectable in RBL 2H3 cells but the detection was less obvious in Jurkat and HEK 293 cells (Fig. 3, B and C). Furthermore, a prominent expression of STIM1 was detected in RBL 2H3 and Jurkat cells and to a lesser extent in HEK 293 cells (Fig. 3G). Concerning the expression of ORAI2, HEK 293 and RBL 2H3 cells provide therefore different backgrounds for the analysis of recombinant channels formed by transfected ORAI1. To define a threshold that allows the detection of ionic currents through recombinant channels, we quantified the background CRAC currents in these cell lines. CRAC current activation was induced by dialysis of 20 μM IP3 under buffer of internal Ca²⁺ with 10 mM EGTA in the presence of 5 mM external Ca²⁺. As
it turned out extremely difficult to obtain similar current recordings in control HEK 293 cells. In fact, only 4 out of 13 HEK 293 cells showed inward rectifying currents in the present series of experiments (Fig. 4, A–D). Next, we overexpressed STIM1 in HEK 293 and RBL 2H3 cells to determine the maximal CRAC current densities that the endogenously expressed ORAI proteins can support in these cell lines. The overexpression of STIM1 failed to enhance significantly the density of inward currents in HEK 293 cells (Fig. 5A) but doubled the CRAC current densities in RBL2H3 cells (Fig. 5B). Therefore, HEK 293 cells represent a cell background, in which CRAC currents are hardly detectable even after overexpression of STIM1. CRAC currents of RBL 2H3 cells appear to be readily modulated by the overexpression of STIM1. Under the present experimental conditions, the threshold levels for detection of CRAC currents through recombinant ionic channels formed

were repeated at least twice. and HEK 293 (lane 2), and RBL 2H3 (lane 3) and probed with the full-length cDNAs encoding ORAI1 (A) and ORAI2L (D). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. The affinity-purified antibodies readily detect the ORAI1 (lane 2) and the ORAI2 protein (lane 2) in COS cells (5 μg of cell lysates), which had been transfected with the respective cDNA but not in non-transfected COS cells (5 μg of cell lysates, lanes 1 in B and E). Arrows indicate monomeric ORAI proteins that run slightly faster as the expected calculated Mr of 33,062 (ORAI1) and 29,802 (ORAI2L) on the blotted 15% separating SDS-PAGE. Slower running proteins might represent dimers and multimers of ORAI because they are not detectable in non-transfected cells (lanes 1). C and F, Western blot of equal amounts of microsomal membrane protein fractions (150 μg per lane) prepared from HEK 293 (lane 2), RBL 2H3 (lane 3), and Jurkat (lane 4) and 5 μl of protein lysates of ORAI-transfected COS cells (lanes 1) as controls were separated on Tricine-SDS-PAGE and probed with the antibodies for ORAI1 and ORAI2. Arrows indicate positions of ORAI1 (C) and ORAI2 (F). G, the antibody for STIM1 detects the endogenously expressed STIM1 protein (G, lane 1) in protein lysates (10 μl) of HEK 293 cells; it recognizes much better the STIM1 protein in the same amount of protein lysate (10 μl) obtained from HEK 293 cells, which had been transfected with the murine STIM1 cDNA (G, lane 2). Using equal amounts of microsomal membrane protein fractions (100 μg per lane) prepared from RBL 2H3 (lane 3), Jurkat (lane 4), and HEK 293 (lane 5), the antibody readily recognizes the endogenously expressed rat (lane 3) and human STIM1 proteins (lanes 4 and 5), which appear to be more abundant in RBL 2H3 and Jurkat than in HEK 293. Western blots were repeated at least twice.

FIGURE 3. Northern (A, D) and Western blots (B, C, E–G) to analyze endogenous expression of ORAI1 (A–C), ORAI2 (D–F), and STIM1 (G) in the cell lines HEK 293, Jurkat, and RBL 2H3. For Northern blots (A, D) poly(A)⁺ RNA was isolated from the cell lines (5 × 10⁶ cells each) HEK 293 (lane 1), Jurkat (lane 2), and RBL 2H3 (lane 3) and probed with the full-length cDNAs encoding ORAI1 (A) and ORAI2L (D). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. The affinity-purified antibodies readily detect the ORAI1 (lane 2) and the ORAI2 protein (lane 2) in COS cells (5 μg of cell lysates), which had been transfected with the respective cDNA but not in non-transfected COS cells (5 μg of cell lysates, lanes 1 in B and E). Arrows indicate monomeric ORAI proteins that run slightly faster as the expected calculated Mr of 33,062 (ORAI1) and 29,802 (ORAI2L) on the blotted 15% separating SDS-PAGE. Slower running proteins might represent dimers and multimers of ORAI because they are not detectable in non-transfected cells (lanes 1). C and F, Western blot of equal amounts of microsomal membrane protein fractions (150 μg per lane) prepared from HEK 293 (lane 2), RBL 2H3 (lane 3), and Jurkat (lane 4) and 5 μl of protein lysates of ORAI-transfected COS cells (lanes 1) as controls were separated on Tricine-SDS-PAGE and probed with the antibodies for ORAI1 and ORAI2. Arrows indicate positions of ORAI1 (C) and ORAI2 (F). G, the antibody for STIM1 detects the endogenously expressed STIM1 protein (G, lane 1) in protein lysates (10 μl) of HEK 293 cells; it recognizes much better the STIM1 protein in the same amount of protein lysate (10 μl) obtained from HEK 293 cells, which had been transfected with the murine STIM1 cDNA (G, lane 2). Using equal amounts of microsomal membrane protein fractions (100 μg per lane) prepared from RBL 2H3 (lane 3), Jurkat (lane 4), and HEK 293 (lane 5), the antibody readily recognizes the endogenously expressed rat (lane 3) and human STIM1 proteins (lanes 4 and 5), which appear to be more abundant in RBL 2H3 and Jurkat than in HEK 293. Western blots were repeated at least twice.

FIGURE 4. Overexpression of the murine ORAI1, ORAI2L, ORAI2S, GAMMA1, and GAMMA2 in two different cell backgrounds. HEK 293 (A–D) and RBL 2H3 (E–H) cells were transfected either with STIM1 alone (S1) or with STIM1 plus ORAI1 (S1 + O1), ORAI2L (S1 + O2L), ORAI2S (S1 + O2S), GAMMA1 (S1 + G1), and GAMMA2 (S1 + G2). Transfected cells that did not express the transfection marker GFP as well as non-transfected cells were used as controls (CTRL). Voltage clamp ramps from +80 to −100 mV were delivered every 2 s to elicit ionic currents. Inward current densities were monitored at −90 mV. The activation of ionic currents was induced with IP₃ (20 μM) under buffer of internal Ca²⁺ (10 mM EGTA) and in the presence of 5 mM external Ca²⁺. The time courses of inward currents densities (A, C, E, G) show the average development of ionic currents in 4–15 cells per transfection protocol. For clarity, the time courses display only every third data point. The current-voltage relationships (B, D, F, H) show representative recordings obtained 40 s after break-in. The transfection protocols are indicated close to each trace.
CRAC current densities that were comparable with those of cells co-expressing additionally ORAI2L and ORAI2S (Fig. 5B), suggesting that the overexpressed ORAI2 variants might not form CRAC channels in this cell line, which expresses endogenous ORAI2 proteins (Fig. 3).

Similarly to ORAI proteins (2–4), the GAMMA subunits of voltage-dependent Ca\(^{2+}\) channels contain four transmembrane domains (23, 24). Therefore, we co-expressed GAMMA 1 and GAMMA 2 with STIM1 in HEK 293 and RBL 2H3 cells (Fig. 4, C, D, G, and H), to test whether these Ca\(^{2+}\) channel subunits interfere with function of STIM1 in forming CRAC channels. As for the individual overexpression of STIM1, co-expression of GAMMA 1 and GAMMA 2 with STIM1 enhanced CRAC current densities significantly in RBL 2H3 cells (Fig. 5B) but not in HEK 293 cells (Fig. 5A). However, the CRAC currents densities measured in the latter co-expression experiments were similar to those observed after the individual overexpression of STIM1 (Fig. 5B), suggesting that the overexpression of GAMMA1 and GAMMA2 does not interfere with the function of CRAC channels. In further experiments, we also explored the effects of transfecting ORAI1, ORAI2L, and ORAI2S individually. Definitely, the endogenous CRAC currents were not enhanced by the individual overexpression of these ORAI proteins in either HEK 293 nor in RBL 2H3 cells (Fig. 5). Due to the low CRAC current densities, possible inhibitory effects were not detectable in HEK 293 cells (Fig. 5A). By contrast, the overexpressed ORAI1, ORAI2L, and ORAI2S slightly inhibited the endogenous CRAC currents of RBL 2H3 cells (Fig. 5B), suggesting that ORAI2L and ORAI2S as well as ORAI1 interact with endogenous CRAC channels in these cells.

The experiments with HEK 293 cells overexpressing STIM1 and ORAI1, ORAI2L, or ORAI2S suggested that densities of CRAC currents strongly depended on the transfected ORAI isoform (Fig. 5A). In these experiments, we observed additionally an inactivation of CRAC currents, which was more pronounced in cells overexpressing ORAI1 plus STIM1 than in those cells transfected with STIM1 and ORAI2 variants (Fig. 4A). Therefore, we investigated whether the different levels of CRAC current densities shown in Fig. 5A reflected differences in the Ca\(^{2+}\)-dependent inactivation that has been previously described for endogenous CRAC currents (21, 22). In this series of experiments, we used BAPTA instead of EGTA in the pipette solution. Under these conditions, almost no inactivation of CRAC currents was observed in cells expressing STIM1 plus ORAI1 or ORAI2 variants (Fig. 6, A, C, and E), suggesting that the current decay observed in the experiments with EGTA reflected a Ca\(^{2+}\) dependent inactivation process. To evaluate the amount of CRAC current inactivation (\(\Delta I_{\text{peak}}\)) in the individual cells, we subtracted the current densities measured 80 s after break-in from the peak current densities (\(I_{\text{peak}}\)). Fig. 6B shows that there is a direct relationship between \(\Delta I_{\text{peak}}\) and \(I_{\text{peak}}\) in the presence of EGTA in cells overexpressing STIM1 and ORAI1, indicating that the amount of current inactivation is proportional to the CRAC current densities in the individual cells.

BAPTA shifted the \(\Delta I_{\text{peak}}-I_{\text{peak}}\) relationship to higher \(I_{\text{peak}}\) values in such a way that CRAC current inactivation was detectable at \(I_{\text{peak}}\) higher than approximately 30 pA/pF (Fig. 6B). In
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the majority of cells with $I_{\text{peak}}$ smaller than 30 pA/pF, the inactivation of CRAC currents was almost abolished by BAPTA. A similar analysis of the CRAC current inactivation in cells overexpressing STIM1 plus ORAI2L suggested that BAPTA reduced almost completely the inactivation of CRAC currents in these cells, probably because CRAC current densities higher than 30 pA/pF were never recorded in these cells (Fig. 6D). Interestingly, no statistical difference was observed between the peak current densities recorded with EGTA and the plateau current densities recorded with BAPTA in cells overexpressing STIM1 and ORAI1 or ORAI2L (Fig. 6, A and C). In cells overexpressing STIM1 and ORAI2S, however, the CRAC current densities measured with BAPTA were 2.6–3.5-fold larger than those recorded with EGTA (Fig. 6E). This effect of BAPTA brought the CRAC current densities of cells overexpressing STIM1 and ORAI2S (Fig. 6E) closer to those recorded with EGTA in cells overexpressing STIM1 and ORAI1 (A, n = 12), ORAI2L (C, n = 11), and ORAI2S (E, n = 10) and dialyzed with BAPTA are shown superimposed on time courses observed in cells dialyzed with EGTA (same experiments as in Fig. 4A). The bath solution contained 5 mM Ca$^{2+}$. The amount of CRAC current inactivation ($\Delta I_{\text{peak}}$) was measured in individual cells as the peak current density ($I_{\text{peak}}$) subtracted by the current density obtained 80 s after break-in. $\Delta I_{\text{peak}}$ is plotted versus $I_{\text{peak}}$ for cells transfected with STIM1 plus ORAI1 (B), ORAI2L (D), and ORAI2S (F). Symbols represent individual cells dialyzed either with EGTA (open symbols) or BAPTA (closed symbols), and lines are approximated fittings to the data.

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Since ORAI2L and ORAI2S failed to enhance CRAC current densities above the levels induced by the overexpression of STIM1 in RBL2H3 cells (Fig. 5), we further tested whether these ORAI2 variants modify the capability of endogenous CRAC channels to conduct Na$^+$ currents in RBL2H3 cells. Fig. 7A illustrates that the exposure to DVF enhanced the inward current densities reflecting the influx of Na$^+$ ions ($I_{\text{Na}}$) through CRAC channels (21, 22). Subsequent perfusion of the Ca$^{2+}$-containing solution showed the anomalous mole fraction effect and exposure to a NCaF abolished completely the current flow, indicating that the main charge carrier of the ionic currents ($I_{\text{Ca}}$) recorded in the Ca$^{2+}$-containing solution were Ca$^{2+}$ ions. Similar responses to the exposure of DVF and NCaF solutions were observed in RBL2H3 cells overexpressing STIM1 plus ORAI2L (Fig. 7B) or ORAI2S (data not shown). Furthermore, we measured the ratios $I_{\text{Na}}/I_{\text{Ca}}$ to determine whether the proportionality between Na$^+$ and Ca$^{2+}$ current densities is modified by the overexpression of STIM1 plus ORAI isoforms. Both in control cells as well as in cells expressing STIM plus ORAI2L or ORAI2S, the average $I_{\text{Na}}/I_{\text{Ca}}$ ratio was in the range of 2–4 (Fig. 7C), indicating that the overexpression of the ORAI2 variants did not modify the capability of endogenous CRAC channels to conduct Na$^+$ currents in the RBL2H3 cells. Similar
results were obtained in RBL2H3 cells overexpressing STIM1 plus ORAI1 (Fig. 7C).

Cells overexpressing ORAI2L and ORAI2S displayed CRAC current densities lower than those overexpressing ORAI1 independently of whether internal Ca\(^{2+}\) was chelated with EGTA or BAPTA (Figs. 5 and 6). Since it has been shown that ORAI proteins form heteromultimers (16), we tested in HEK 293 cells whether ORAI2 variants influence the functional expression of ORAI1 in triple transfection experiments (Fig. 8). The plasmid ratio STIM1:ORAI1 was 1:2 (Figs. 4A and 5A), and low amounts of plasmids containing ORAI2 variants were added to obtain the triple combinations STIM1:ORAI1:ORAI2L and STIM1:ORAI1:ORAI2S at a ratio of 1:2:1. Accordingly, cells transfected with STIM1 and ORAI1 were used as controls. Notably, the CRAC current densities of cells transfected with STIM1, ORAI1, and ORAI2S were lower than those transfected only with STIM1 and ORAI1, indicating that ORAI2S might interfere with the functional expression of ORAI1 (Fig. 8A). The maximal current densities of cells transfected with STIM1, ORAI1, and ORAI2L were nearly identical to those overexpressing STIM1 and ORAI1. The current-voltage relations recorded in the triple transfection experiments were indistinguishable from those observed in cells overexpressing STIM1 and ORAI1 (Figs. 4B and 8B). Additionally, the time course of CRAC current inactivation appeared to be slower in the triple transfected cells (Fig. 8A), but a possible effect of the ORAI2 variants on CRAC current inactivation was not further analyzed.

DISCUSSION

Recent experiments have implicated ORAI and STIM proteins in the formation of CRAC channels. So far, most studies of ORAI and STIM proteins have been carried out either with human or Drosophila isoforms. Here we report the cloning, expression patterns, and functional expression of the murine orai orthologs. Interestingly, the murine orai2 gene encodes at least two different proteins that differ in their N termini and are functional expressed in a cell background dependent manner. Variants similar to ORAI2L appear not to be expressed in humans.

The human orai1, orai2, and orai3 have been localized in chromosomes 12, 7, and 16, respectively (2). We have localized the murine orai1 and orai3 genes as single copies on chromosome 5G1 and 7F2-F3, respectively. However, we found copies of orai2 on chromosomes 5G2 and 16C1. The orai2 locus on chromosome 5 contains at least five exons and four introns, while the locus on chromosome 16 represents an intronless gene containing only three exons (Fig. 1A). Likely, the orai2 locus on chromosome 16 resulted from viral integration by involvement of reverse transcription of the original transcript 3, which contains the encoding sequence of exons 1, 4, and 5 from the locus on chromosome 5 (Fig. 1A). Similar events were discussed for several genes (25), including the intronless calmodulin gene in chicken (26) and the brain-4 class III POU-domain gene Pou3f4 in mice (27). Interestingly, copies of orai2 were also found in the rat chromosomes 4 and 12. Thus, it appears that duplicated copies of orai2 are common in rodents. The present information on the genomic loci of orai2 may become important when considering the generation of mouse deletion mutants. However, the question arises on whether transcripts of orai2 are generated in mice from both chromosomes 5 and 16. We found that three different transcripts arise from the locus on chromosome 5 due to alternative splicing events (Fig. 1A).

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STIM1 and ORAI1 were found to be expressed broadly in mammalian tissues (2, 11, 16). In the present series of experiments, we detected also a ubiquitous expression of ORAI1 and STIM1 in mice tissues (Fig. 2). Furthermore, we show that ORAI3 is expressed broadly in murine tissues, but the expression pattern of the ORAI2 variants appears to be more restricted. More interestingly, we observed a prominent expression of ORAI2 variants in the murine brain (Fig. 2). Since the transcripts encoding ORAI2L and ORAI2S (Fig. 1A) were amplified from murine brain, both ORAI2 variants are likely co-expressed in this tissue. Additionally, we found that ORAI2L and ORAI2S are also co-expressed as independent transcripts...
in murine T lymphocytes, B cells, and mast cells (supplemental Fig. 3). Since the human ORAI1 and ORAI2 form functional CRAC channels in conjunction with STIM1 (8–10), the expression patterns shown in Fig. 2 suggest that CRAC channels might be ubiquitously expressed in mammalian cells. Although CRAC currents have been extensively studied in hematopoietic cells, however, detailed studies of CRAC currents in non-hematopoietic cells are still hampered by the fact that the current densities are close to the levels of current detection (21, 22). Since STIM1 alone amplifies endogenous CRAC currents in HEK 293 and Jurkat cells but ORAI1 alone does not (8–10), a likely explanation for the low CRAC current densities in non-hematopoietic cells may be that the levels of STIM1 expression limit the formation of functional CRAC channels in these cells. Alternatively, STIM2, the only known isoform of STIM1, may also play a dominant negative role because overexpression of STIM2 inhibits CRAC currents (28). However, the expression levels of ORAI and STIM proteins have not been determined in the cell lines commonly used in previous overexpression studies. Using specific antibodies, we found that ORAI1 and ORAI2 proteins are hardly detectable in HEK 293 cells (Fig. 3). This observation is in line with the fact that CRAC currents are hardly detectable in HEK 293 cells (Figs. 4 and 5). By contrast, RBL 2H3 cells show a detectable expression of ORAI1, ORAI2, and STIM1 (Fig. 3). In overexpression experiments, we next sought to analyze the functional expression of the ORAI isoforms in the HEK 293 and RBL 2H3 cell backgrounds, which differ both in the levels of endogenous ORAI and STIM expression and in their responses to the STIM1 overexpression (Figs. 3–5). Independently of the cell background, ORAI1 expressing cells displayed prominent CRAC currents as described previously (8–10). We also observed that the overexpression of the ORAI2 variants induced CRAC current densities smaller than those induced by ORAI1 under identical conditions in HEK 293 cells (Fig. 5). Since STIM1 was co-expressed in these experiments, the reason for the different levels of functional expression of ORAI1 and ORAI2 variants might rely on their capability to interact with STIM1. Furthermore, the CRAC current densities of RBL 2H3 cells overexpressing ORAI2 variants were similar to those overexpressing STIM1 alone (Fig. 5). Thus, the capability of ORAI2L and ORAI2S to form recombinant CRAC channels depends also on the cell background, in which these proteins are expressed. Additionally, we explored the influence of internal Ca\(^{2+}\) on the CRAC currents of transfected cells. Comparing the effects of the Ca\(^{2+}\) chelators EGTA and BAPTA on the inactivation of CRAC currents in HEK 293 cells overexpressing STIM1 plus ORAI1 or ORAI2 variants, we found that the CRAC channels formed by these ORAI isoforms undergo a Ca\(^{2+}\)-dependent inactivation, which is proportional to the levels of CRAC current density and, thus, to the levels of recombinant CRAC channels expressed in the individual cells (Fig. 6). Accordingly, the degree of CRAC current inactivation, and its inhibition by BAPTA was also dependent on the cell individual levels of recombinant CRAC channel expression. The inhibition of CRAC current inactivation by BAPTA had, however, low impact on the maximal current densities of cells overexpressing ORAI1 and ORAI2L but enhanced approximately 2.5-fold the CRAC current densities in those cells overexpressing ORAI2S (Fig. 6). Thus, it appears that ORAI2S is more sensitive to inactivation by internal Ca\(^{2+}\) than ORAI2L and ORAI1. In triple transfection experiments with STIM1, ORAI1, and ORAI2S, we recorded CRAC current densities that were lower than in the transfection experiments with only STIM1 and ORAI1 (Fig. 8). Such effect was not seen in cells transfected with STIM1, ORAI1, and ORAI2L. Thus, it appears that ORAI2S might play a dominant negative role in the formation of functional CRAC channels by ORAI1. Since the main difference between ORAI1 and ORAI2S resides in the length of the N terminus (supplemental Fig. 1), our results suggest an important role for the N terminus of ORAI1 proteins in the formation and function of CRAC channels. In support of this suggestion, it has been recently shown that over-expression of the N terminus of ORAI1 inhibits the capacitative Ca\(^{2+}\) entry in HEK 293 cells (29).

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