THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 40, pp. 34830–34838, October 7, 2011

Selenoprotein K Is a Novel Target of m-Calpain, and Cleavage Is Regulated by Toll-like Receptor-induced Calpastatin in Macrophages*[S]

Received for publication, May 26, 2011, and in revised form, July 28, 2011 Published, JBC Papers in Press, August 17, 2011, DOI 10.1074/jbc.M111.265520

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Background: Selenoprotein K is important for calcium-dependent activation of immune cells.
Results: Selenoprotein K is cleaved by m-calpain in resting macrophages, but Toll-like receptor activation induces calpastatin generating full-length, functional selenoprotein K.
Conclusion: Proteolytic modulation of selenoprotein K is important for macrophage activation.
Significance: New roles are defined for the calpain/calpastatin system and selenoprotein K during macrophage activation and inflammation.

Calpains are proteolytic enzymes that modulate cellular function through cleavage of targets, thereby modifying their actions. An important role is emerging for calpains in regulating inflammation and immune responses, although specific mechanisms by which this occurs have not been clearly defined. In this study, we identify a novel target of calpain, selenoprotein K (SelK), which is an endoplasmic reticulum transmembrane protein important for Ca^{2+} flux in immune cells. Calpain-mediated cleavage of SelK was detected in myeloid cells (macrophages, neutrophils, and dendritic cells) but not in lymphoid cells (B and T cells). Both m- and μ-calpain were capable of cleaving immunoprecipitated SelK, but m-calpain was the predominant isoform expressed in mouse immune cells. Consistent with these results, specific inhibitors were used to show that only m-calpain cleaved SelK in macrophages. The cleavage site in SelK was identified between Arg^{81} and Gly^{82} and the resulting truncated SelK was shown to lack selenocysteine, the amino acid that defines selenoproteins. Resting macrophages predominantly expressed cleaved SelK and, when activated through different Toll-like receptors (TLRs), SelK cleavage was inhibited. We found that decreased calpain cleavage was due to TLR-induced up-regulation of the endogenous inhibitor, calpastatin. TLR-induced calpastatin expression not only inhibited SelK cleavage, but cleavage of another calpain target, talin. Moreover, the expression of the calpain isoforms and calpastatin in macrophages were different from T and B cells. Overall, our findings identify SelK as a novel calpain target and reveal dynamic changes in the calpain/calpastatin system during TLR-induced activation of macrophages.

Calpains are calcium-activated cysteine proteases that cleave specific targets to modulate cellular functions such as apoptosis, proliferation, and migration. An important role is emerging for calpains in regulating inflammation and immune responses, although specific mechanisms by which this occurs have not been clearly defined (1–3). There are two major isoforms of this enzyme, μ-calpain (or calpain I) and m-calpain (or calpain II), which require micromolar and millimolar Ca^{2+} concentrations for activity, respectively (4). These enzymes are comprised of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. Activation occurs after Ca^{2+} binding induces conformational changes that lead to autocleavage of the N-terminal inhibitory domain of the 80 kDa subunit (5, 6). Because the activation of calpain is an irreversible reaction, its activity must be tightly regulated by mechanisms in addition to fluctuating Ca^{2+} levels. A key part of this regulation is calpastatin, which is an endogenous inhibitor of calpain. Calpastatin contains four tandem repeats of a calpain inhibitory domain and each calpastatin is capable of inhibiting more than one calpain molecule (7).

The endoplasmic reticulum (ER)[2] membrane has been identified as an important site of calpain/calpastatin association and activity. In early studies, calpain and calpastatin were identified in the ER isolated from A549 lung adenocarcinoma cells (8). Topological studies suggested that m-calpain was oriented with the catalytic 80-kDa subunit mostly exposed to the cytolosic side of the ER membrane, along with the regulatory regions of calpastatin (9). Interactions between m-calpain and calpastatin were subsequently found to occur on the cytosolic, not luminal, side of the ER membrane (10). This is important given the stores of Ca^{2+} in the ER lumen may be released into the lumen during activation of various types of cells, particularly immune cells. Thus, proteins localized to the ER membrane or newly synthesized proteins emerging from the ribosomal machinery

* This research was supported, in whole or in part, by National Institutes of Health Grants R21AT004844 and R01AI089999.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.
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[2] The abbreviations used are: ER, endoplasmic reticulum; BMDM, bone marrow-derived macrophages; GPx, glutathione peroxidase; IC, immune complex; RANKL, receptor activator of NF-κB ligand; SelK, selenoprotein K; TLR, Toll-like receptor; Txnrd, thioredoxin reductase.

at the surface of the rough ER may be potential targets for calpain/calpastatin proteolytic modulation.

Selenoproteins are a family of proteins that contain the 21st amino acid, selenocysteine (11). There are 25 selenoproteins in humans, 24 of which are also found in mice. Selenoproteins serve a variety of biological functions including regulation of thyroid hormone metabolism, intra- and extracellular antioxidation, redox regulation, protein retrotranslocation from the ER to cytoplasm, and sperm maturation/protection (12). Our laboratory recently identified selenoprotein K (SelK) as an ER membrane protein important for Ca^{2+} flux during the activation of immune cells (13). T cells, neutrophils, and macrophages from SelK$^{-/-}$ mice exhibited decreased receptor-mediated Ca^{2+} flux in response to several different stimuli and were functionally impaired in terms of migration, proliferation, and cytokine secretion. The expression of SelK is relatively high in immune tissues and sensitive to levels of dietary Se intake (13). Thus, SelK represents an important mechanism by which dietary Se influences inflammation and immunity and a better understanding of its regulation is required.

There appear to be common features of the calpain/calpastatin system and SelK. Both have been shown to be important for cellular migration, including immune cell chemotaxis (13–15). Subcellular localization is also similar with SelK localized to ER membranes, where the calpain/calpastatin network is operable. The selenocysteine-containing region of SelK is localized to the cytosolic side of the ER membrane (16), placing it in proximity to the catalytic domain of m-calpain. These facts in addition to multiple isoforms detected for SelK in certain immune cell-types led us to investigate the possibility that SelK serves as a target for calpain cleavage. In the current study we confirm that SelK is a substrate for m-calpain, with proteolysis producing a truncated isoform of SelK lacking the selenocysteine residue. The cleaved isoform is highly abundant in unactivated macrophages and, upon activation with several different Toll-like receptor ligands, calpastatin expression is up-regulated and inhibits m-calpain cleavage and leads to an increase in full-length, selenocysteine-containing SelK. These data provide major insight into actions and regulation of the calpain/calpastatin system in a major cell-type involved in inflammation.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6J wild-type controls were generated from mice originally purchased from the Jackson Laboratory. Generation of SelK$^{-/-}$ mice was previously described (13). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Antibodies and Reagents—Antibodies for Western blots included anti-calpastatin (Cell Signaling), anti-talin and anti-m-calpain (Santa Cruz Biotechnology), anti-μ-calpain (Millipore), anti-β-actin (Sigma), and anti-α-tubulin (Novus Biologicals). Two anti-SelK antibodies were used, including a polyclonal against the C-terminal portion (Sigma) and a monoclonal against the N-terminal portion (Epigenomics). Secondary antibodies were purchased from Li-Cor Technologies. LPS (0111:B4) was purchased from Sigma, CpG from Trilinker, and poly(i:c) and zymosan from InVivogen. Inhibitors were purchased against μ-calpain (Santa Cruz Biotechnology), m-calpain (EMD Chemicals), and both isoforms of calpain (Sigma). Low avidity immune complexes (IC) were constructed by incubating 20 μg of BSA (Invitrogen) with 200 μg of anti-BSA (Upstate/Millipore) in 500 μl of PBS (4:1 molar ratio anti-BSA to BSA). After 1 h of incubation at room temperature, the low avidity IC was stored at 4 °C until use. High avidity IC were constructed using 1.0 μm fluorescent, carboxylate-modified microspheres (Molecular Probes/Invitrogen) coated with BSA. Anti-BSA (10 μg from Upstate/Millipore) was added to 2×10^9 BSA-coated beads in 100 μl of PBS and incubated for 2 h. These IgG-opsonized beads were washed with PBS and centrifugation as above and counted by hemacytometer prior to use. Monocyte chemoattractant protein-1 (MCP-1) was purchased from R&D Systems.

Plasmids and Transfections—The cDNA encoding full-length mouse SelK was synthesized by Genscript and subcloned into the expression plasmid, pcDNA3.1(+) (Invitrogen). We then subcloned SelK from this plasmid into pEGFP-C1 (Clontech/BD Biosciences). Using these two plasmids containing full-length SelK, inverse PCR was performed to generate plasmids encoding truncated SelK and EGFP-tagged truncated SelK. All expression plasmids were transfected into SelK KO BMDM using a Neon transfection system (Invitrogen).

Preparation of ex Vivo Cells and BMDM—Purification of T cells, B cells, and dendritic cells from spleen and neutrophils from bone marrow was performed using a Miltenyi magnetic separator and purity of cells was determined by evaluation of each cell-type marker via flow cytometry on a FACScaliber (BD Biosciences) and found to be >90%. For bone marrow-derived macrophages (BMDM), marrow was flushed from femurs and tibiae with HBSS using a syringe with a 25-gauge needle and cell suspensions were then passed through a 40-μm pore cell strainer (BD Falcon) to remove tissue debris. The cells were plated in DMEM containing 10% FCS, 1% penicillin/streptomycin/L-glutamine (Invitrogen), and 10% L929 conditioned media and used on day 6 of culture.

^{75}Se Labeling and Immunoprecipitations—BMDM were plated in complete media and on day 5 pulsed with ^{75}Se (4 μCi/ml) in the absence or presence of 100 ng/ml LPS. After 24 h, cells were lysed, an aliquot of the whole cell lysate stored at −20 °C, and remaining whole cell lysates were incubated with 15 μl of protein-A Dynabeads (Invitrogen) with 10 μg of pre-bound monoclonal anti-SelK for 2 h to immunoprecipitate SelK. The beads were washed and incubated at 95 °C for 10 min in laemmli buffer and autoradiography performed as previously described (17). HEK293 cells were transfected with this plasmid or empty control plasmid using Lipofectamine 2000 (Invitrogen) for 24 h before lysis and analysis for protein expression. Associations between calpastatin, m-calpain and SelK were detected by a ligand affinity purification assay method (18, 19). Anti-m-calpain antibody (10 μg) was cross-linked to Dynabeads using 5 mM Bis (sulfosuccinimidyl) suberate (Thermo Fisher), and beads were incubated with BMDM at 70 rpm for 30 min at 37 °C. Dimethyl 3,3′-dithiobispropionimidate-2HCl (DTBP from Thermo Fisher) was added to bead-bound cells to a final concentration of 3 mM, and samples incubated for another 30 min at 37 °C (70 rpm). The cross-linking reaction was quenched with 20 mM Tris-HCl before isolation of bead-

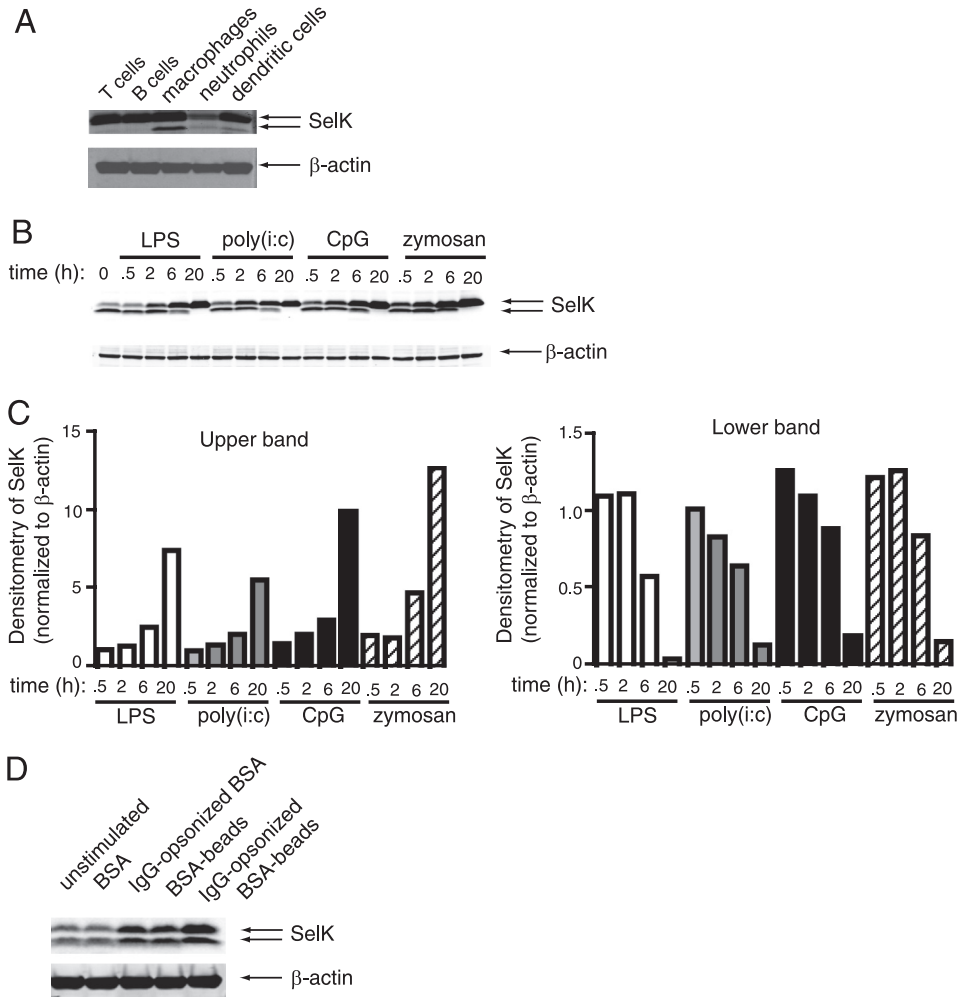

FIGURE 1. **Two TLR-regulated isoforms of SelK are detected in myeloid cells.** *A*, primary lymphoid (T and B cells) and myeloid (macrophages, neutrophils, and DCs) cells were analyzed by Western blot to detect SelK. *B* and *C*, BMDM were unstimulated (0 h) or stimulated (0.5–20 h) with different TLR ligands. SelK isoforms were detected by Western blot and densitometry performed. *D*, BMDM were stimulated through FcγR for 20 h with low avidity immune complexes (*IgG-opsonized BSA*) with BSA as a control, or stimulated with high avidity immune complexes (*IgG-opsonized BSA beads*) with BSA beads as a control. Loading control for all blots was β-actin.

bound cells on a magnet. Samples were washed with CSK buffer (10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.8), 50 mM NaCl, 150 mM sucrose, 3 mM $MgCl_2$, and 1 mM $MnCl_2$) supplemented with 20 mM Tris-HCl (pH 8.5) and 2 mM Na_3VO_4. Cells were lysed in CSK^+ buffer (CSK buffer supplemented with 0.5% (w/v) Triton X-100, EDTA free protease inhibitor mixture) for 30 min on ice with sonication. Beads were separated and washed 3 times with CSK^+ buffer. Antibody enriched m-calpain and associated calpastatin or SelK were eluted from beads with 1× reducing sample buffer (Bio-Rad). Protein samples were separated from beads with a magnet, resolved by SDS-PAGE, and then analyzed by Western blot.

In Vitro Calpain Cleavage Assays—HEK293 cells were transfected with pcDNA3.1(+)-SelK and overexpressed SelK protein immunoprecipitated from lysates as described above. After three washes, the beads were resuspended in calpain buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 30 mM NaCl, 5 mM DTT. 1 μg of human μ-calpain (Athens Research & Technology) or porcine m-calpain (EMD Chemicals) was added, and the reaction incubated at 37 °C for 1 h, with 50 mM EDTA added to stop the reaction. The cleavage products were separated from the reaction buffer using 10 kDa cut-off centricon filters (Millipore), and analyzed by NanoLC-MS/MS (Applied Biomics). Laemmli buffer was then added to the beads and Western blot performed to identify SelK isoforms after cleavage. In other experiments, 0.5 mg of synthetic custom peptide from Peptide 2.0 (PPGNPPRRMGRISHLRGPSPPPMAGGCGR) was incubated with 1 μg of porcine m-calpain in calpain reaction buffer for 2 h. The reaction was then passed through a 10 kDa cut-off filters to remove the m-calpain and the resulting products analyzed by NanoLC-MS/MS (Applied Biomics).

Ca^{2+} Flux and Migration Assays—BMDM from $SelK^{-/-}$ mice were transfected with plasmids and stimulated with LPS for 24 h prior to assays. Measurement of Ca^{2+} flux was measured using fluo4 (Invitrogen) as previously described (13), with 100 ng/ml MCP-1 added to initiate Ca^{2+} flux. Migration assays were performed using 8 μm CytoSelect 24-well Cell Migration Assay kits (Cell Biolabs). BMDM (1×10^6 cells) were added to upper chambers in 200 μl of serum-free medium. Lower chambers contained 500 ml of complete RPMI 1640 medium with 100 ng of MCP-1 added as a chemoattractant. Migration was carried out for 8 h at 37 °C and 10% CO_2, and number of migrat-

ing cells determined using CyQuant GR fluorescent dye and a standard curve per manufacturer's instructions.

Statistical Analyses—Means between groups were compared using Graphpad Prism 4, which was also used for regression curve fitting.

RESULTS

Isoforms of SelK in Myeloid Cells Are Regulated by TLR Ligands—We previously demonstrated a relatively high level of SelK expression in spleen and other lymphoid tissues (13), and this led us to investigate which immune cells express this selenoprotein. Interestingly, lymphoid cells (T and B cells) exhibited one band for SelK while two bands were detected in myeloid cells (macrophages, neutrophils, and dendritic cells) (Fig. 1*A*). The lower band was particularly evident in macrophages. We hypothesized that the presence of two SelK isoforms may be related to different activation states of the myeloid cells. Thus, we used bone marrow-derived macrophages (BMDM) to investigate how activation through different Toll-like receptors (TLRs) affects abundance of the two different SelK isoforms. BMDM were stimulated with ligands for TLR2 (zymosan), TLR3 (poly(i:c)), TLR4 (LPS), and TLR9 (CpG) in a time-course manner and the effects on SelK isoforms examined. All four TLR ligands produced similar changes in SelK expression involving decreases in the smaller isoform with concordant increases in the larger isoform (Fig. 1, *B* and *C*). This switch in SelK isoforms was evident as early as 6 h after stimulation and only the upper band was detectable after 20 h post-stimulation. To determine if other types of receptor stimulation produced similar effects on SelK isoforms, BMDM were stimulated through the Fcγ receptors using different immune complexes. Fcγ receptor stimulation did not produce changes in different SelK isoforms, and instead led to equivalent increases in both isoforms (Fig. 1*D*).

Cleavage by m-Calpain Is Responsible for the Two SelK Isoforms—SelK has been suggested to be transcriptionally upregulated during ER stress (20). However, we found that TLR ligands did not increase SelK mRNA levels nor did they induce ER stress (supplemental Figs. S1 and S2), suggesting that TLR ligands act on SelK expression by mechanisms other than through ER stress signals. We hypothesized that different SelK isoforms may arise from post-translational regulation and, after ruling out various modes of protein modification (*e.g.* phosphorylation, glycosylation, ubiquitinylation), we turned our attention to proteolysis. Using immunoprecipitated SelK and purified μ- and m-calpain, we found that both calpains cleaved full-length SelK from LPS-stimulated BMDM to produce a smaller band of equal size to that detected in untreated, resting BMDM (Fig. 2*A*). More complete cleavage was found with m-calpain. We repeated this approach using full-length SelK overexpressed in HEK293 cells, and m-calpain was again more effective at cleaving SelK (Fig. 2*B*). We found that treatment of resting macrophages with m-calpain inhibitors, but not μ-calpain inhibitors, produced similar effects to LPS treatment in that the smaller SelK isoform was absent (Fig. 2*C*). Interestingly, both LPS and the m-calpain inhibitor prevented cleavage of another target of calpain, talin. This suggests that the effects of TLR ligands on calpain proteolysis are not specific to SelK,

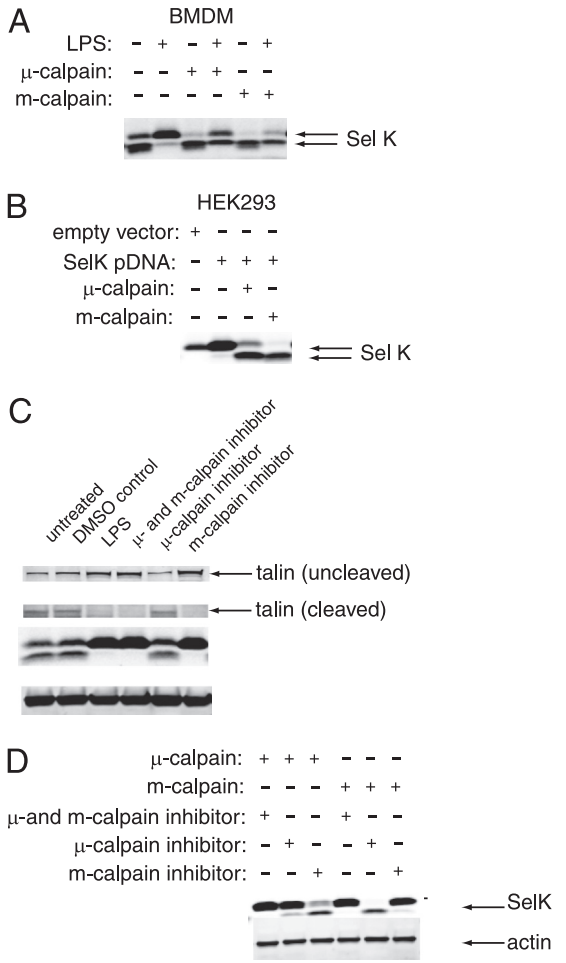

FIGURE 2. **Calpain cleavage generates the two SelK isoforms.** *A*, SelK was immunoprecipitated from BMDM lysates and purified μ- or m-calpain used to cleave full-length SelK and produce a band of the same size as that found in unstimulated BMDM. *B*, full-length SelK was overexpressed in HEK293 cells and cleavage with μ- or m-calpain produced a smaller isoform of similar size to that found in unstimulated BMDM. *C*, BMDM were treated with LPS or with inhibitors for both or each μ- or m-calpain for 20 h. Western blot analysis demonstrated that calpain cleavage of both SelK and talin were similarly inhibited by LPS and the m-calpain inhibitor. Loading control was β-actin. *D*, SelK was immunoprecipitated from BMDM lysates and purified μ- or m-calpain used to cleave full-length SelK in presence of each calpain inhibitor. Results confirm specificity of each inhibitor for appropriate calpain isoform.

but may inhibit cleavage of many calpain targets. To verify specificity of each inhibitor, immunoprecipitated SelK was cleaved with purified calpain isoforms in combination with inhibitors (Fig. 2*D*).

Identification of the Calpain Cleavage Site in SelK—The location of the selenocysteine residue near the C terminus of the SelK protein allowed the use of ^{75}Se incorporation into this amino acid followed by autoradiography to determine if the C-terminal portion of SelK was present in both the full-length and cleaved SelK isoforms. Results demonstrated that both full-length and truncated SelK are immunoprecipitated, but selenocysteine was detected only in full-length SelK and was absent in the cleaved isoform (supplemental Fig. S3). This confirmed the small isoform does not include the C-terminal portion, and the size difference between the two isoforms suggested the calpain cleavage site is located ~1000–1500 Da from the C terminus of SelK. Using the CaMPDB web-based program (21), we found

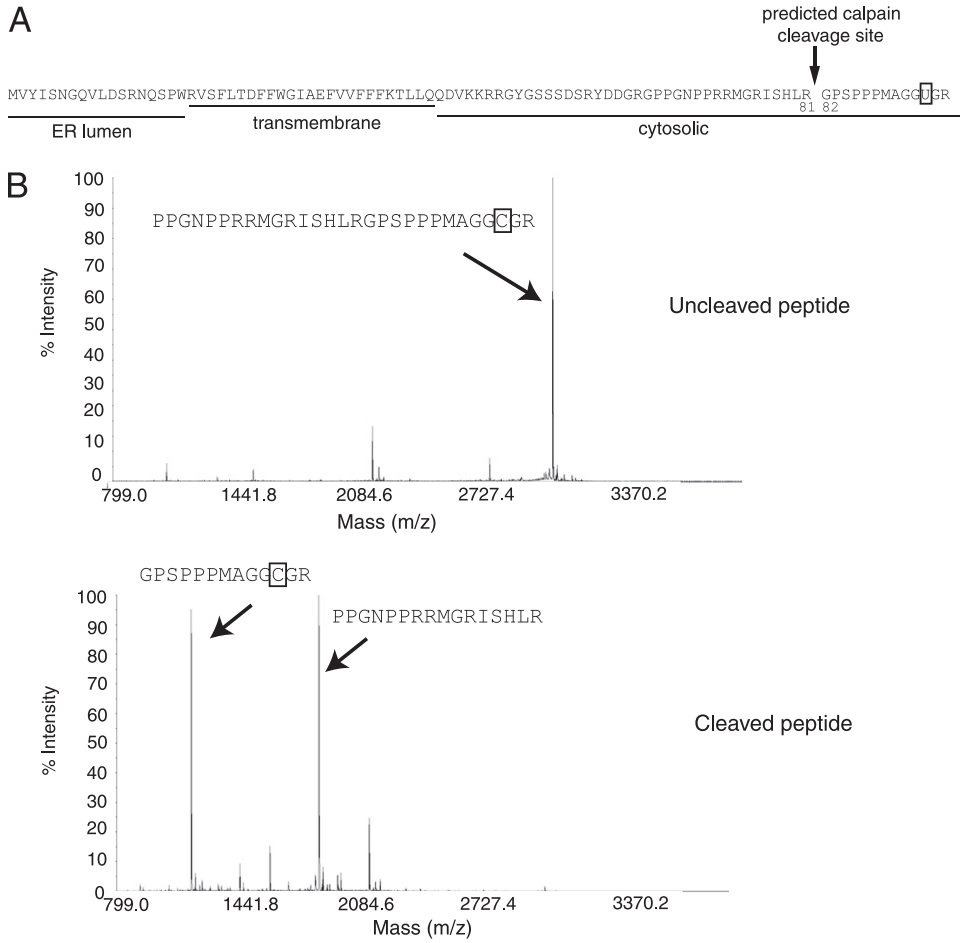

FIGURE 3. **Identification of the calpain cleavage site in SelK.** *A* and *B*, web-based program (18) was used to predict calpain cleavage sites in SelK, with the highest scored cleavage site located at $Arg^{81} \downarrow Gly^{82}$. Selenocysteine is represented by U and highlighted with a *box*. *C*, synthetic peptide consisting of the 29 C-terminal amino acids of SelK with Cys (*boxed C*) in place of selenocysteine was cleaved by m-calpain and products identified by mass spectrophotometry. Uncleaved peptide was sequenced as a control and shown in *upper panel*.

that the highest scoring predicted cleavage site in SelK was located between Arg^{81} and Gly^{82} residues (Fig. 3*A*). Cleavage of SelK at this site would produce an isoform ~1350 Da smaller than full-length lacking selenocysteine, which is consistent with our ^{75}Se labeling results above. We next conducted *in vitro* cleavage to investigate the precise location of the calpain cleavage site in SelK. A synthetic peptide comprising the 29 amino acids in the C-terminal portion of SelK was cleaved with purified m-calpain and resulting peptides analyzed by mass spectrophotometry. Calpain cleavage produced two products and amino acid sequencing confirmed cleavage of SelK at the $Arg^{81} \downarrow Gly^{82}$ site (Fig. 3*B*). This experiment was repeated by cleaving immunoprecipitated SelK with purified m-calpain and the same cleavage site was identified (supplemental Fig. S4). Importantly, the synthetic peptide was generated with Cys in place of selenocysteine and was effectively cleaved by calpain, indicating selenocysteine is not required for calpain cleavage.

Calpastatin Is Up-regulated by LPS Resulting in Full-length SelK—The results indicating that m-calpain cleaves SelK in unactivated macrophages but not in activated macrophages are somewhat counterintuitive. In particular, Ca^{2+} levels typically increase during activation and increased Ca^{2+} should activate, not inhibit, calpain (22). This led us to consider regulators of calpain activity other than Ca^{2+} levels. Another key regulator of calpain is the endogenous inhibitor, calpastatin, and we found that LPS-treatment increased calpastatin levels in BMDM (Fig. 4, *A* and *B*). LPS-induced increases in calpastatin were detected as early as 30 min and peaked at 20 h post-stimulation concurrent with decreased SelK and talin cleavage. Furthermore, stimulation through all four TLR ligands increased calpastatin, which corresponded to decreased cleavage of both SelK and talin (Fig. 4*C*). We performed co-immunoprecipitations and found that SelK associated with m-calpain prior to LPS-stimulation, but this was decreased with LPS treatment, which coincided with m-calpain/calpastatin association (Fig. 4*D*). Given the differences in SelK isoforms shown in Fig. 1 between lymphocytes and myeloid cells, we again examined T and B cells compared with macrophages regarding levels of calpastatin. In contrast to macrophages, T cells constitutively expressed calpastatin and had no cleavage of either talin or SelK (Fig. 4*E*). Interestingly, B cells expressed low levels of calpastatin but showed no detectable cleavage of either talin or SelK. Thus, we performed Western blot analyses for μ- and m-calpain expression and found that B cells expressed very little m-calpain compared with T cells and macrophages (Fig. 4*F*). Interestingly, there was no detectable expression of μ-calpain in any of these three mouse immune cells. Overall, there appeared to be quite

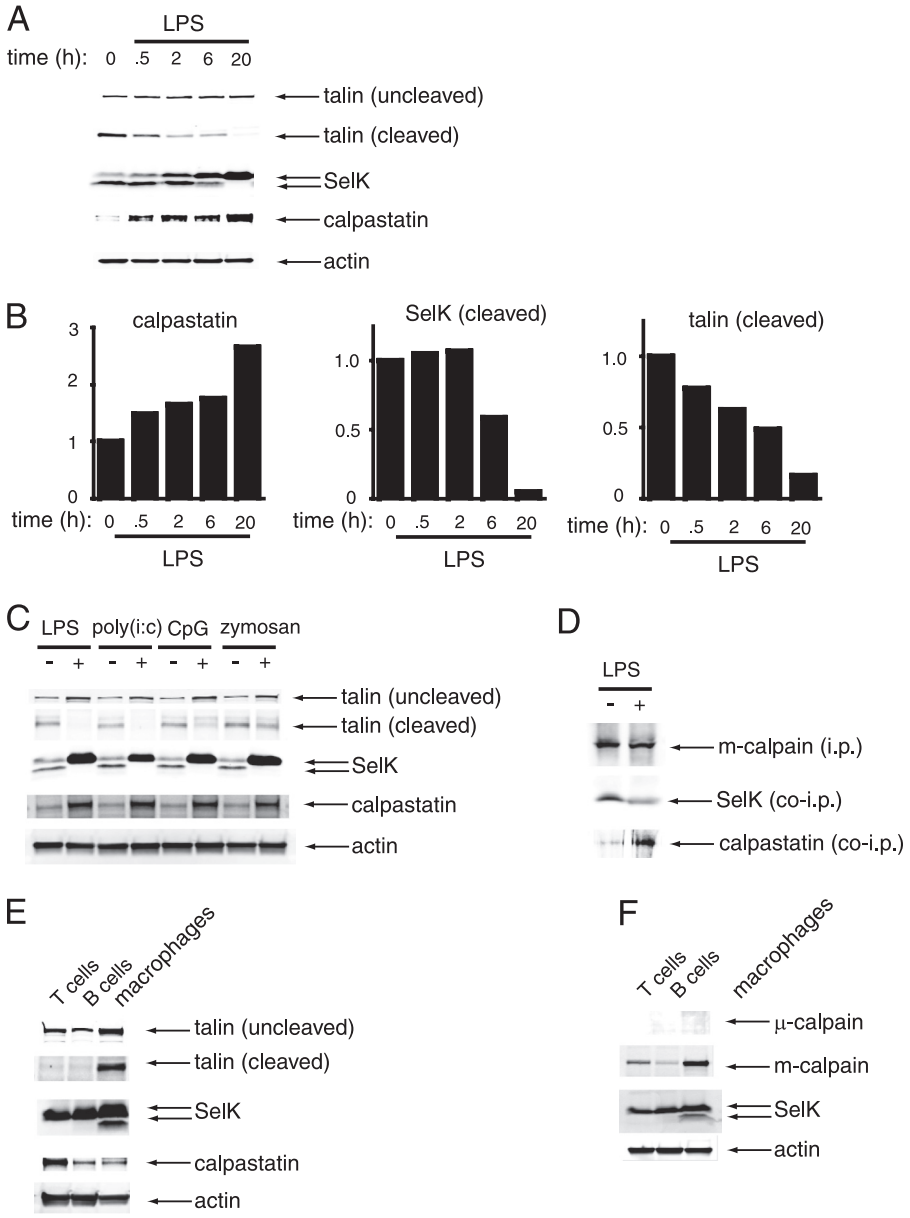

FIGURE 4. **Calpastatin is up-regulated by TLR ligands.** *A* and *B*, BMDM were treated with LPS for increasing time and Western blot used to detect levels of calpastatin, uncleaved and cleaved talin, and isoforms of SelK. Densitometry was performed showing increases in calpastatin over time that coincided with decreases in both cleaved SelK and talin. *C*, BMDM stimulated for 20 h with four different TLR ligands exhibited increased calpastatin and decreased cleavage of both SelK and talin. *D*, T cells, B cells, and macrophages from mice were analyzed by Western blot for levels of calpastatin and cleavage of SelK and talin. *E*, Western blot detection of μ- and m-calpain expression in T cells, B cells, and macrophages (the 80 kDa catalytic subunits) showed that m-calpain was the only detectable isoform and was mainly expressed in T cells and macrophages. Loading control for all blots was β-actin. *F*, m-calpain was immunoprecipitated from BMDM and either m-calpain, Sel K, or calpastatin detected by immunoblotting. Results show much more SelK co-immunoprecipitation prior to LPS-treatment, but calpastatin immunoprecipitation after 20 h LPS treatment.

different expression patterns of m-calpain and calpastatin in the different immune cell types.

Full-length SelK Is Required for Effective Ca^{2+} Flux and Migration—Our results indicated that m-calpain cleavage of SelK is inhibited in activated macrophages and SelK has been shown to promote Ca^{2+} flux (13). Thus, we investigated whether full-length SelK was functionally superior to truncated SelK. BMDM from $SelK^{-/-}$ mice were rescued with vector control, or vectors encoding full-length or truncated SelK, and cells stimulated with LPS. During transfection, these cells were stimulated with LPS and after 30 h both Ca^{2+} flux and migratory capacity in response to monocyte chemoattractant protein-1 (MCP-1) were evaluated. Results showed that full-length SelK functioned for MCP-1-induced Ca^{2+} flux and promoted optimal migration (Fig. 5, *A* and *B*). Western blot was used to confirm equivalent expression of both full-length and truncated SelK and EGFP-tagged versions of these proteins showed similar expression patterns in the rescued cells (Fig. 5, *C* and *D*).

DISCUSSION

The modulation of cellular function through the calpain cleavage system involves three main protein components: calpain, its substrates, and calpastatin. There are more than 100 substrates for calpain cleavage and identifying new targets is an

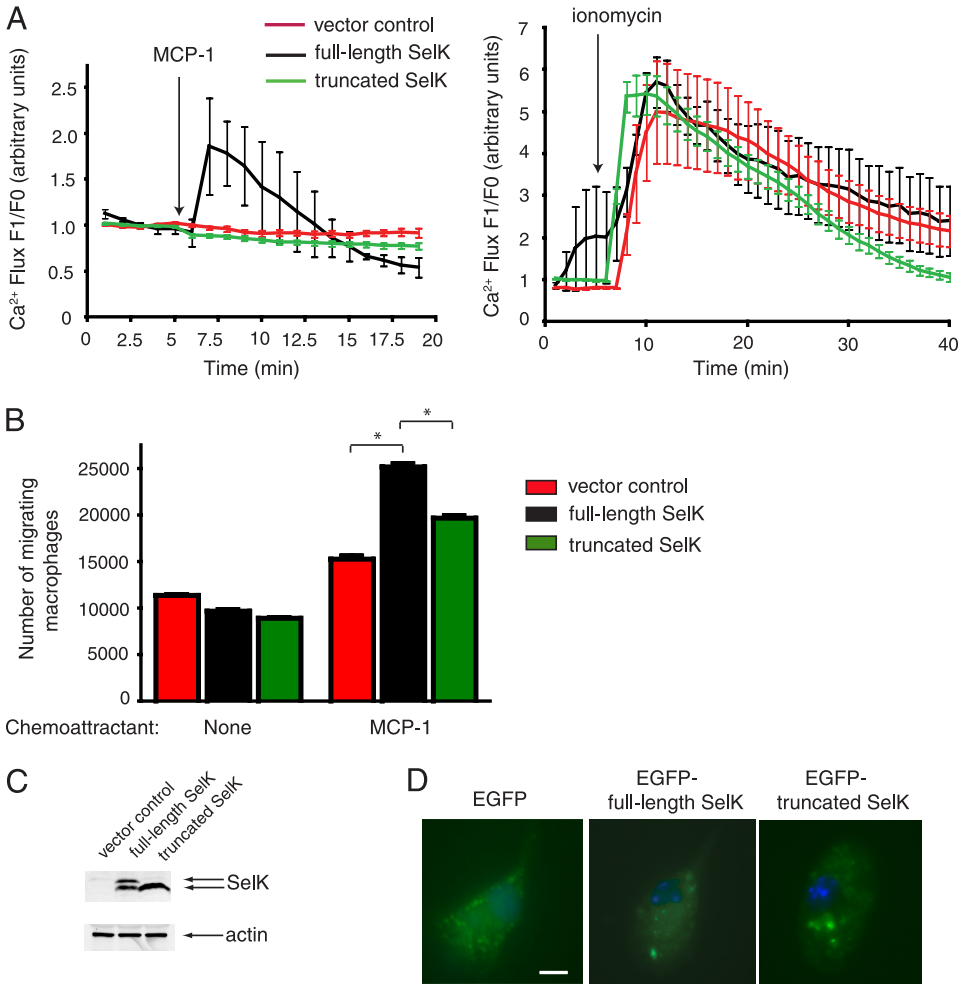

FIGURE 5. **Full-length SelK, but not truncated SelK, is functionally active.** BMDM from SelK$^{-/-}$ mice were transfected with control vector, or a vector encoding full-length or truncated SelK. *A*, After 40 h, these different BMDM were evaluated for Ca^{2+} flux in response to MCP-1 stimulation. *B*, transfected BMDM were also examined for their ability to migrate in response to MCP-1. For *A* and *B*, results represent mean ± S.E. ($n = 3$) and means were compared using a Student's *t* test. *C*, Western blot analysis shows equivalent expression of full-length or truncated SelK. *D*, in separate experiments, BMDM from SelK$^{-/-}$ mice were transfected with EGFP-expression control vector, or a vector encoding EGFP-tagged full-length or truncated SelK. The EGFP was diffuse, while EGFP tagged to both full-length and truncated SelK were excluded from nuclei with expression patterns similar to each other. Nuclei were stained blue with DAPI and scalebar = 5 μm.

important step toward understanding how this system affects cellular processes and how best to therapeutically target the calpain/calpastatin system. We have identified SelK as a novel target of m-calpain, which is the major calpain isoform expressed in T cells and macrophages. In macrophages, calpastatin is induced through TLR stimulation in macrophages to prevent its cleavage (Fig. 6). Calpastatin appears to be constitutively expressed in other cell types and controls the actions of calpain for not only SelK, but also for other targets of calpain cleavage such as talin. Thus, expression of the calpains as well as their endogenous inhibitor, calpastatin, represents an important system of proteolytic modulation that exhibits different expression between different types of immune cells. There is a small amount of full-length SelK in resting macrophages, and our previously published work shows that this is important for the TLR stimulation of resting macrophages (13). Overall, the data presented herein suggest that the increase in full-length SelK after TLR activation is for signaling in activated macrophages when they are stimulated through receptors that induce Ca^{2+} flux. This is supported by data in Fig. 5, and rapid Ca^{2+} flux in activated macrophages requires full-length SelK.

Calpain proteolyzes target proteins in a limited manner rather than completely digesting them. This often serves to modulate functions of the substrate proteins by cutting their interdomain regions (23, 24). Our data indicate that calpain cleavage of SelK occurs at the predicted site (. . . $R_{81} \downarrow G_{82}$ . . .) to generate a truncated SelK isoform lacking the selenocysteine residue. The selenocysteine residue is the distinguishing feature of selenoproteins and most often centrally involved in their biological functions. For example, the glutathione peroxidases (GPx) and thioredoxin reductases (Txnrd) are subsets of selenoprotein enzymes that utilize selenocysteine at their catalytic site to carry out oxidoreductase reactions (12). SelK lacks defined redox motifs found in antioxidant selenoproteins like GPx and Txnrd enzymes and it remains unclear exactly what role the selenocysteine serves in SelK. However, our data suggest that the presence of the selenocysteine residue in SelK is regulated by the calpain/calpastatin system during the activation of macrophages.

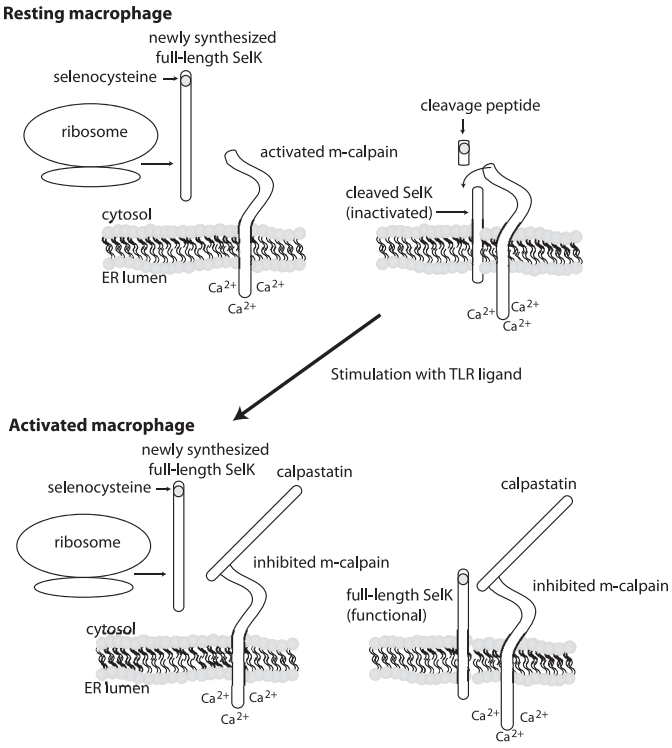

FIGURE 6. **Model for the effects of TLR stimulation on the calpain/calpastatin system and SelK cleavage.** In resting macrophages, newly synthesized SelK is immediately cleaved by m-calpain upon insertion into the ER membrane. Upon stimulation with various TLR ligands, calpastatin expression is induced and inhibits the cleavage of SelK. Cleavage of talin and perhaps other calpain targets are similarly regulated by this mechanism.

Our findings suggest that calpain-mediated cleavage of target proteins is altered as resting macrophages differentiate into an activated phenotype. Calpain may play a key role in the differentiation process as signaling and cytoskeletal elements are proteolytically altered to promote the activation process. For example, migratory capacity is different between resting and activated macrophages, and cytoskeletal proteins involved in cell motility are regulated by calpain cleavage, including talin, focal adhesion kinase, and paxillins (14, 25–27). The assembly and disassembly of focal adhesion complexes are regulated by calpain cleavage, with important adaptor molecules such as paxillin and others serving as calpain targets (28). Given our previous findings implicating SelK in the migration of T cells and neutrophils (13), inhibiting calpain cleavage may serve to generate an active, selenocysteine-containing isoform of SelK involved in focal adhesion and cell motility. In fact, cytoskeletal dynamics are crucial for several processes in activated macrophages including migration, phagocytosis, secretion of cytokines, and antigen presentation (29). Our findings that the calpain/calpastatin system is altered during TLR-induced activation of macrophages may provide new insight into the proteolytic control of cytoskeletal dynamics and further investigation into their influences on these functions is warranted.

In addition to cytoskeleton dynamics, other signaling pathways are regulated by both μ- and m-calpain. For example, addition of receptor activator of NF-κB ligand (RANKL) to RAW 264.7 mouse macrophages caused an increase in calpain activity (30). In these studies, μ-calpain was essential for NF-κB activation in macrophages induced by RANKL. Another study used a mouse model of hypercholesterolemic nephropathy to demonstrate that macrophages stimulated through the nicotinic acetylcholine receptor α1 induced μ-calpain activation and inflammatory signaling (31). Perhaps most relevant to our findings with SelK are those by Ueda *et al.* showing that m-calpain regulates α-crystallins, which are chaperone proteins related to small heat shock family of proteins (30). Similar to our findings with SelK, m-calpain cleavage of α-crystallins removes 11 amino acids from the C terminus of the target protein, which inactivates its function. In fact, SelK may itself act as a chaperone to coordinate protein-protein interactions at the surface of ER and thus modulate a wide range of cellular functions including cytoskeletal dynamics, Ca^{2+} flux from the ER, transport of misfolded proteins from the ER, and promoting cell signaling. Studies are currently in progress to determine how the truncated *versus* full-length SelK isoforms may functionally differ.

There appears to be no consensus calpain cleavage sequence and this has slowed progress of identification of targets of this enzyme. Most likely, calpain recognizes the overall three-dimensional structure of its substrates more than the primary structure. However, there are amino acid preferences at positions surrounding calpain cleavage sites, with amino acids designated as P_4-P_1 $\downarrow$ P_1'-P_7' (32). There has been proposed a P_2-P_1 rule, which states that the preferred residues for calpain are Leu and Val at position P_2 and Arg or Lys at P_1 (4, 33, 34). Pro dominates the region flanking the P_2-P_1' segment, particularly at P_3' (35). Our data show that SelK follows the P_2-P_1 rule, but contains a Pro at the P_2' and P_4' positions instead of P_3'. Delineation of preferential amino acid sequences encompassing calpain cleavage sites is important for development of inhibitors, and calpain has been suggested to be an attractive target of therapeutic inhibitors for treatment of various inflammatory disorders (12, 36). Development of such therapies will require a full characterization of the targets, cell types in which the calpain/calpastatin system operates, and modes by which proteolytic modulation through this system is regulated.

Acknowledgments—The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCCAM, NIAID, or National Institutes of Health.

REFERENCES

1. Deshpande, R. V., Goust, J. M., Chakrabarti, A. K., Barbosa, E., Hogan, E. L., and Banik, N. L. (1995) *J. Biol. Chem.* **270,** 2497–2505
2. Hendry, L., and John, S. (2004) *Eur. J. Biochem.* **271,** 4613–4620
3. Svensson, L., McDowall, A., Giles, K. M., Stanley, P., Feske, S., and Hogg, N. (2010) *PLoS One* **5,** e15090
4. Brown, N., and Crawford, C. (1993) *FEBS Lett.* **322,** 65–68
5. Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K., and Bode, W. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 588–592
6. Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z., and Davies, P. L. (2002) *Cell* **108,** 649–660
7. Wendt, A., Thompson, V. F., and Goll, D. E. (2004) *Biol. Chem.* **385,** 465–472
8. Hood, J. L., Brooks, W. H., and Roszman, T. L. (2004) *J. Biol. Chem.* **279,** 43126–43135
9. Samanta, K., Kar, P., Ghosh, B., Chakraborti, T., and Chakraborti, S. (2007)

Biochim. Biophys. Acta **1770,** 1297–1307
10. Samanta, K., Kar, P., Chakraborti, T., Shaikh, S., and Chakraborti, S. (2010) *J. Biochem.* **147,** 765–779
11. Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V., Zehtab, O., Guigó, R., and Gladyshev, V. N. (2003) *Science* **300,** 1439–1443
12. Reeves, M. A., and Hoffmann, P. R. (2009) *Cell Mol. Life Sci.* **66,** 2457–2478
13. Verma, S., Hoffmann, F. W., Kumar, M., Huang, Z., Roe, K., Nguyen-Wu, E., Hashimoto, A. S., and Hoffmann, P. R. (2011) *J. Immunol.* **186,** 2127–2137
14. Franco, S. J., and Huttenlocher, A. (2005) *J. Cell Sci.* **118,** 3829–3838
15. Letavernier, E., Dansou, B., Lochner, M., Perez, J., Bellocq, A., Lindenmeyer, M. T., Cohen, C. D., Haymann, J. P., Eberl, G., and Baud, L. (2011) *Eur. J. Immunol.* **41,** 473–484
16. Chen, C. L., Shim, M. S., Chung, J., Yoo, H. S., Ha, J. M., Kim, J. Y., Choi, J., Zang, S. L., Hou, X., Carlson, B. A., Hatfield, D. L., and Lee, B. J. (2006) *Biochem. Biophys. Res. Commun.* **348,** 1296–1301
17. Reeves, M. A., Bellinger, F. P., and Berry, M. J. (2010) *Antioxid. Redox. Signal* **12,** 809–818
18. Plopper, G., and Ingber, D. E. (1993) *Biochem. Biophys. Res. Commun.* **193,** 571–578
19. Humphries, J. D., Byron, A., Bass, M. D., Craig, S. E., Pinney, J. W., Knight, D., and Humphries, M. J. (2009) *Sci. Signal* **2,** ra51
20. Du, S., Zhou, J., Jia, Y., and Huang, K. (2010) *Arch. Biochem. Biophys.* **502,** 137–143
21. duVerle, D., Takigawa, I., Ono, Y., Sorimachi, H., and Mamitsuka, H. (2010) *Genome Inform* **22,** 202–213
22. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) *Physiol. Rev.* **83,** 731–801
23. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) *Biochem. J.* **328,** 721–732
24. Saido, T. C., Shibata, M., Takenawa, T., Murofushi, H., and Suzuki, K. (1992) *J. Biol. Chem.* **267,** 24585–24590
25. Carragher, N. O., Levkau, B., Ross, R., and Raines, E. W. (1999) *J. Cell Biol.* **147,** 619–630
26. Franco, S., Perrin, B., and Huttenlocher, A. (2004) *Exp. Cell Res.* **299,** 179–187
27. Chan, K. T., Bennin, D. A., and Huttenlocher, A. (2010) *J. Biol. Chem.* **285,** 11418–11426
28. Cortesio, C. L., Boateng, L. R., Piazza, T. M., Bennin, D. A., and Huttenlocher, A. (2011) *J. Biol. Chem.* **286,** 9998–10006
29. Fettucciari, K., Quotadamo, F., Noce, R., Palumbo, C., Modesti, A., Rosati, E., Mannucci, R., Bartoli, A., and Marconi, P. (2011) *Cell Microbiol.* **13,** 859–884
30. Lee, F. Y., Kim, D. W., Karmin, J. A., Hong, D., Chang, S. S., Fujisawa, M., Takayanagi, H., Bigliani, L. U., Blaine, T. A., and Lee, H. J. (2005) *J. Biol. Chem.* **280,** 29929–29936
31. Zhang, G., Thomas, A. L., Marshall, A. L., Kernan, K. A., Su, Y., Zheng, Y., Takano, J., Saido, T. C., and Eddy, A. A. (2011) *Lab. Invest.* **91,** 106–123
32. Ueda, Y., Fukiage, C., Shih, M., Shearer, T. R., and David, L. L. (2002) *Mol. Cell Proteomics* **1,** 357–365
33. Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilágyi, A., Bánóczi, Z., Hudecz, F., and Friedrich, P. (2004) *J. Biol. Chem.* **279,** 20775–20785
34. Stabach, P. R., Cianci, C. D., Glantz, S. B., Zhang, Z., and Morrow, J. S. (1997) *Biochemistry* **36,** 57–65
35. Carragher, N. O. (2006) *Curr. Pharm. Des.* **12,** 615–638
36. Pietsch, M., Chua, K. C., and Abell, A. D. (2010) *Curr. Top Med. Chem.* **10,** 270–293