TRPM2 is a cation channel unique within the transient receptor potential family because of its gating by ADP-ribose (ADPR). ADPR gating is enabled by a cytosolic C-terminal Nudix box sequence motif embedded into a region homologous to the NUDT9 ADPR pyrophosphatase. A recently discovered splice variant of TRPM2 (TRPM2-ΔC) lacks 34 amino acid residues in the NUDT9 domain and is insensitive to ADPR. To analyze in detail which parts of the deleted sequence (ΔC-stretch) are critical for ADPR gating, we tested mutants that lacked 19, 25, and 29 amino acid residues in the N-terminal part or had amino acid residues substituted in the remaining C-terminal part of the ΔC-stretch. All of these mutants displayed typical ADPR-induced currents. However, the deletion or substitution of the amino acid residue Asn-1326 immediately downstream of the N-terminal domain of TRPM2-ΔC has been attributed to the reverse exchange EF → IL. This region has sequence homology to the human NUDT9 ADPR pyrophosphatase (12). The refined crystal structure analysis as well as biochemical studies indicate that the human NUDT9 enzyme can be functionally divided into a N-terminal domain of 105 amino acid residues, which possibly supports ADPR binding and a 179-amino-acid residue C-terminal domain containing the catalytic active site (12, 13).

The enzymatic activities of the human NUDT9 ADP-Rase and the isolated and purified TRPM2 NUDT9-H domain in vitro were intensively studied (7, 14). Accordingly, a substitution of two neighboring amino acid residues (mutation EF → IL) in the TRPM2 Nudix box accounts for a decreased enzymatic activity of the NUDT9-H domain in comparison to NUDT9. However, the importance of any residual enzymatic activity of NUDT9-H for the ADPR gating of TRPM2 remains unclear. It may be hypothesized that the binding of ADPR to the NUDT9-H domain rather than degradation of ADPR by the NUDT9-H domain is decisive for channel gating. Moreover, although ADPR-binding seems to be supported by the N-terminal part of NUDT9, it is poorly understood which structures within this region govern the interaction with ADPR.

Recently, we have identified a C-terminal splice variant of TRPM2 (TRPM2-ΔC) in neutrophil granulocytes. This variant is insensitive to ADPR but develops typical current concentrations in the presence of H₂O₂ (15). The corresponding deletion of 34 amino acid residues (the ΔC-stretch) is located within the N-terminal domain of TRPM2 NUDT9-H (13).

The aim of the present study was to analyze in detail which parts of the ΔC-stretch are critical for the ADPR gating of TRPM2. Furthermore, we analyzed the impact on channel function when the Nudix box of TRPM2 was changed such that the two amino acid residues essential for the full enzymatic activity are the same as in the NUDT9 pyrophosphatase. We report that the substitution of these two amino acid residues of TRPM2 abrogates stimulation by ADPR, but no individual amino acid residue of the ΔC-stretch is essential for channel gating. However, the deletion or substitution of an asparagine residue immediately downstream of the ΔC-stretch completely abolished ADPR-gating.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning—For expression in eukaryotic cells the plasmid construct pCDNA3-EGFP containing the full-length open reading frame of TRPM2 was used. For the generation of TRPM2 mutants with C-terminal deletions similar to the TRPM2-ΔC splice variant, as described by (15), several BspDI recognition sites were introduced in pairs into the TRPM2 open reading frame. Site-directed mutagenesis was performed, using the QuikChange mutagenesis system (Stratagene) and defined oligonucleotides (see Table I) obtained from MWG-Biotech AG (Ebersberg, Germany). Subsequently, the DNA (pCDNA3-EGFP-
TRPM2 was digested with BspDI (New England Biolabs, Beverly, MA) yielding two DNA fragments. After separation on a 1% agarose gel the excised small DNA fragment was discarded, and the large DNA fragment with the corresponding deletion was isolated using the QiaQuick gel extraction kit (Qiagen, Hilden, Germany) and circularized using the Quick Ligation kit (New England Biolabs). The resulting TRPM2 expression constructs contain open reading frames with defined deletions between 19 and 39 amino acids in length. Every point mutation or deletion was verified by DNA sequencing with the BigDyeTerminator Kit (PerkinElmer Life Sciences). The presence of inadvertent mutations in other regions of the channel could be excluded, because for each deletion mutant or point mutant two clones were tested yielding the same results. All procedures were performed in accordance to the respective manufacturers’ instructions, if not indicated otherwise.

Cell Culture and Transfection—Chinese ovary hamster cells K1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in Ham’s F12 medium (Biochrome, Berlin, Germany) supplemented with 4 mM l-glutamine and 10% (v/v) fetal calf serum (Biochrome). Cells were seeded on glass coverslips at a density of <10^5 cells/mm² and grown for 24 h. Subsequently, the pcDNA3-EGFP-TRPM2/TRPM2ΔC expression constructs were transiently transfected into the Chinese hamster ovary cells, using the Trans-Fast transfection reagent (Promega). As controls, cells were transfected with pcDNA3-enhanced green fluorescent protein vector alone. The transfection procedure was as described elsewhere (15).

Electrophysiology—Transfected cells were analyzed with the patch clamp technique in the whole-cell mode, using a EPC 9 equipped with a personal computer with Pulse and X chart software (HEKA, Lambrecht, Germany). The standard extracellular bath solution contained (in mM) 140 NaCl, 1.2 MgCl₂, 1.2 CaCl₂, 5 KCl, 10 HEPEs, pH adjusted with KOH to 7.4. For Na⁺-free solutions, Na⁺ was replaced by 150 mM N-methyl-D-glucamine, and the titration was performed with HCl. The pipette solution contained (in mM) 145 cesium-glutamate, 8 NaCl, 2 MgCl₂, 10 Cs-EGTA, 10 HEPEs, pH 7.2 (CsOH). When a high (1 µM) intracellular Ca²⁺ concentration was desired, the ETGA concentration in the pipette fluid was reduced to 1 mM, and 0.89 mM CaCl₂ was added. Immediately before measurements, ADP-ribose was added to the pipette solution from a stock, yielding a final concentration of 0.5 mM. In distinct experiments with the mutants N1326D, ΔAsn-1326, and I1405E/L1406F, the ADP-ribose concentration in the pipette solution was increased up to 10 mM. Cells were held at a potential of −60 mV at room temperature. The current-voltage (I-V) relations were obtained during voltage ramps from −90 to +60 mV applied over 400 ms.

RESULTS

In a previous study (15), we have identified a C-terminal splice variant of the cation channel TRPM2 (Δ1292–1325 or TRPM2-ΔC) in which the gating of the channel by ADPR is lost. The deleted sequence of 34 amino acids (the ΔC-stretch) is located within the N-terminal part of a region homologous to the human NUDT9 ADPRase (Ref. 12, see also Fig. 1). To figure out the amino acid residues of the NUDT9-II region critical for the ADPR-sensitivity of TRPM2, we first performed modifications of TRPM2 that allowed us to delete the ΔC-stretch in total or in part. Therefore, we introduced two BspDI recognition sites into the full-length TRPM2 open reading frame local-
ized immediately upstream and downstream of the ΔC-stretch (Fig. 1). After digestion with BspDI we obtained two DNA-fragments, the excised short ΔC-stretch, which was discarded, and the large TRPM2-ΔC expression vector construct lacking the desired 39 amino acids (Δ39); this construct was religated. The obtained deletion is congruent with the original ΔC-stretch but additionally two N-terminal and three C-terminal amino acid residues are lacking. Furthermore, one amino acid codon is changed at each end of the deletion because of the two BspDI recognition sites introduced previously (see Fig. 1).

To test TRPM2 variants functionally, Chinese hamster ovari-K1 cells were transfected with the respective constructs. We did not use human embryonic kidney-293 cells as the expression system, because we had not found activation of TRPM2 currents by NAD in these cells (15), which is contradictory to Ref. 16 and our results obtained in neutrophil granulocytes (17).

The ADPR sensitivity was studied with the patch clamp technique in the whole-cell mode. ADPR (0.3 mM) was applied to the cytosolic side of the channels by dialysis into the cells through the patch pipette. As expected, cells expressing the Δ39 variant of TRPM2 (which is almost identical with the previously characterized ADPR-insensitive TRPM2-ΔC variant) failed to respond to ADPR with a development of currents (Fig. 2 and Table II). The duration of each experiment after

Table II

| Phenotype (n) | ADPR sensitivity | Current density (pA/pF) |
|--------------|------------------|------------------------|
| Wild type (3)  | +                | 150.9 ± 55.2           |
| Δ39 (9)       | −                | 0.38 ± 0.3             |
| Δ19 (3)       | +                | 109.7 ± 68             |
| Δ25 (4)       | +                | 118.3 ± 33             |
| Δ29 (6)       | +                | 77.6 ± 34              |
| Q1320E (3)    | +                | 78.6 ± 54              |
| G1322W (4)    | +                | 141.4 ± 36             |
| P1324Q (3)    | +                | 113.1 ± 19             |
| L1325D (7)    | +                | 51.5 ± 24              |
| L1325G (3)    | +                | 88.4 ± 58              |
| N1326D (15)   | −                | 1.75 ± 1.0             |
| ΔAsn-1326 (13)| −                | 1.87 ± 1.5             |
| P1327V (3)    | +                | 45.0 ± 37              |
| I1405E/L1406F (33)| −         | 1.00 ± 0.9            |
obtaining the whole-cell configuration was at least 5 min to exclude the effects of variable diffusion of ADPR from the pipette into the cytosol.

In the next step we changed the localization of the 3'-BspDI recognition site, which was shifted upstream in steps of 10, 14, and 20 amino acid residues. We obtained three additional TRPM2 channel variants designated Δ29, Δ25, and Δ19 in which the ΔC-stretch was shortened at its C terminus (see Fig. 1).

During infusion of 0.3 mM ADPR into cells expressing these Δ29, Δ25 and Δ19 channels, respectively, an inward current developed rapidly and consistently (Fig. 2 and Table II). The current was characterized by a reversal potential of \( V_r \) and was blocked in the inside direction when the extracellular Na+ was substituted by the large impermeable cation \( N\text{-methyl-D-glucamine} \) (Fig. 4). Thus, these experiments show the typical ADPR-induced non-selective cation currents previously demonstrated characteristic for TRPM2 (7, 8, 15).

Hence, we had excluded for the 28 N-terminal amino acid residues of the ΔC-stretch that their deletion is responsible for the loss of function of TRPM2-ΔC. This left us with a remaining stretch of six amino acids in the C-terminal part of the original ΔC-stretch that might be critical for ADPR gating of the channel (Fig. 1). Their potential importance is implied by the fact that some of these amino acid residues are conserved between TRPM2 and NUDT9 (12). To test their functional relevance, we performed a site-directed mutagenesis in the TRPM2 wild-type channel. The individual amino acid residues were substituted such that their structure or charge were profoundly altered. In particular, we generated four TRPM2 mutants: Q1320E, G1322W, P1324Q, and L1325G (see Fig. 1). Surprisingly, none of these point mutations affected the normal ADPR sensitivity of the channel (Figs. 3 and 4 and Table II). This suggests that no single amino acid residue of the ΔC-stretch is essentially involved in the ADPR gating of TRPM2 channels. (Only for Ala-1321 and Leu-1323 is no information available because these were not mutated). The data imply that the region C-terminally of the ΔC-stretch might be critical. Hence, we next generated the mutations N1326D and P1327V of the full-length TRPM2 that affect the two amino acid residues located immediately downstream of the ΔC-stretch (Fig. 1).

Both these residues are the first ones within a stretch of 12 amino acid residues almost completely conserved between TRPM2 and NUDT9 (Ref. 12, see also Fig. 1). The P1327V channel showed typical cation currents in the presence of ADPR (Fig. 4, Table II). In distinct contrast, the N1326D channel did not display any ADPR-induced activity (Fig. 3, Table II). Even with higher than standard (0.3 mM) ADPR concentrations (1 and 10 mM), no currents were evoked (Table II). To further confirm the importance of Asn-1326, we deleted this amino acid residue. Indeed, the deletion mutant...
Asn-1326 did not display currents in response to ADPR (Fig. 3, Table II). These data and the fact that during the splice process generating TRPM2-ΔC channels an acidic residue (Glu-1291) is transferred immediately upstream of Asn-1326 (15) let us hypothesize that the findings may fit to the simple model, no acidic residue is tolerated in the region around Asn-1326. To test this hypothesis, we studied the mutation L1325D and a construct produced in preparation of the channel-variant Δ39. This construct was identical with full-length TRPM2 but already contained the two BspDI recognition sites, resulting in four amino acid substitutions, notably G1329D (Fig. 1).

**Fig. 4.** Current-voltage relationship of ADPR-induced currents of wild-type (WT) and mutant TRPM2 channels. Currents were recorded in the presence of ADPR (0.3 mM in the pipette) during voltage ramps from −90 to +60 mV, in normal bath (1) or in the presence of N-methyl-D-glucamine (2), to cells expressing wild-type or mutant TRPM2.
Critical Amino Acids for ADP-ribose Activation of TRPM2

As the main finding of the present study, we have identified the amino acid residues within the ΔC-stretch (Δ1292–1325) of wild-type TRPM2 that can be deleted (Δ1292–1318) or mutated (V1319D, Q1320E, G1322W, P1324E, L1325G, L1325D) without disturbing ADPR gating. Thus, although the ΔC-stretch is essential for ADPR-induced channel activation, it most probably contains no amino acid residues that are directly involved in the ADPR gating of TRPM2. In contrast, the amino acid residue Asn-1326 located immediately downstream and therefore outside of the ΔC-stretch is essential, because the substitution of N1326D caused a loss of function.

Indeed, no currents were induced by ADPR in cells transfected with this channel construct (n = 4, data not shown). However, the mutant L1325D displayed normal ADPR-induced channel activity (Fig. 3, Table II), demonstrating that not every acidic substitution close to Asn-1326 causes a loss of function.

It has been shown in vitro that the EF → IL substitution in the Nudix box of the purified NUDT9-H domain of TRPM2 is responsible for the drastic decrease of the enzymatic activity, if compared with human NUDT9 ADPRase (14). To clarify the role of this domain for ADPR gating, we performed the reverse substitution IL → EF in TRPM2, i.e. we reintroduced the two amino acid residues decisive for the high enzymatic activity of NUDT9 (see Fig 5A).

This mutant I1405E/L1406F did not respond to ADPR in the standard concentration of 0.3 mM (Table II). Our assumption that the IL → EF substitution may restore the full ADPR hydrolase activity in vivo implies that the reinforced degradation of the substrate might impair the ADPR gating. Therefore, we performed additional experiments in which the ADPR concentration was considerably increased to 0.6, 0.9, and 10 mM, respectively. Moreover, the intracellular Ca²⁺ concentration was raised from <10 nM to ~1 μM because it was demonstrated by Ref. 18 that the intracellular Ca²⁺ provides essential positive feedback for channel activation. However, even at the highest concentration, no ADPR-induced current was observed at low (Fig. 5B, Table II) or high intracellular Ca²⁺ (data not shown).

Thus, the TRPM2 mutants N1326D, ΔAsn-1326, and I1405E/L1406F are not ADPR-activated channels. This raises the question whether they might develop currents in the presence of other activators. Both wild-type TRPM2 and its functional mutants with substitutions in the ΔC-stretch (exemplary shown for G1322W in Fig. 6) displayed typical currents with NAD (1 mM) in the patch pipette. However, we detected no NAD-dependent channel activation with the ADPR-insensitive mutants N1326D, ΔAsn-1326, and I1405E/L1406F (data not shown).

In a previous study (15) using human embryonic kidney-293 cells as an expression system, we demonstrated characteristic currents through the splice variant TRPM2-3C induced by H₂O₂. Unfortunately, we were not able to demonstrate a H₂O₂ induction of currents in the Chinese hamster ovary expression system using wild-type TRPM2 with a sufficient consistency. Therefore, we did not rigorously test H₂O₂ on mutants insensitive to ADPR.

DISCUSSION

As the main finding of the present study, we have identified the amino acid residues within the ΔC-stretch (Δ1292–1325) of wild-type TRPM2 that can be deleted (Δ1292–1318) or mutated (V1319D, Q1320E, G1322W, P1324E, L1325G, L1325D) without disturbing ADPR gating. Thus, although the ΔC-stretch is essential for ADPR-induced channel activation, it most probably contains no amino acid residues that are directly involved in the ADPR gating of TRPM2. In contrast, the amino acid residue Asn-1326 located immediately downstream and therefore outside of the ΔC-stretch is essential, because the substitution of N1326D as well as the deletion of ΔAsn-1326 disrupts the ADPR gating of the channel.

Recent biochemical studies as well as crystallization analyses with the homologous human NUDT9 ADPRase indicate that the NUDT9-H domain of TRPM2 is composed of a N-terminal part supporting ADPR binding and a C-terminal part containing the Nudix box motif critical for the enzymatic activity (12, 13). The ΔC-stretch, which is deleted in the ADPR-
insensitive splice variant TRPM2-ΔC, is located adjacent to the transition of these two subdomains of NUDT9-H. Interestingly, Asn-1326 represents the N-terminal residue of a sequence of 12 amino acids that is almost completely conserved between TRPM2 NUDT9-H and NUDT9 (with the exception of Met-1328 and Arg-1334, Ref. 12). However, the mutation of further residues within this region showed diverging results. The equally conserved amino acid residue Pro-1327 was changed to valine without a negative effect on ADPR-gating, whereas a construct with the double mutation M1328I/G1329D did not exhibit ADPR-induced currents. Thus, Asn-1326 is a residue of singular importance for the function of TRPM2. The deletion of the ΔC-stretch of the previously described TRPM2-ΔC-splice variant (15) appears to induce localized structural disruptions specifically affecting Asn-1326. Other residues in the vicinity such as Met-1328 and Gly-1329 may also be involved.

TRPM2 represents a channel protein that additionally may possess enzymatic activity. However, the enzymatic activity has not been demonstrated for the complete channel but only for its purified NUDT9 domain (7, 14). Moreover, the enzymatic activity was considerably lower than in the human NUDT9 ADPR pyrophosphatase because of the amino acid exchange E1405F/I1406L. This could be interpreted as an evolutionary adaptation, producing an extended dwell time of ADPR at its binding site, which may be required for channel gating. In this context, it is noteworthy that stimulation of TRPM2 specifically depends on ADPR, whereas degradation products of ADPR are without effect (7). When we performed the reverse substitution I1405E/L1406F in TRPM2, with the idea that any enzymatic activity might be strongly increased, any current induction by ADPR was lost. These data are in line with the hypothesis that the restored ADPRase activity of the TRPM2 Nudix box mutant interfered with the ADPR gating of the channel, because the channel activator was rapidly degraded. One may expect that higher ADPR concentrations can compensate for the degradation. However, this was not the case in our experiments because even with the highest ADPR concentrations and in the presence of elevated intracellular Ca\textsuperscript{2+} concentrations (that facilitate ADPR gating, Ref. 18), no current development was observed. Thus, if we assume that the mutation I1405E/L1406F does not disturb normal channel assembly and/or trafficking, we conclude that ADPR gating of TRPM2 does not tolerate manipulations that enhance the putative ADPR pyrophosphatase activity of the Nudix box.

Because we observed the complete loss of ADPR-dependent channel activity for the mutants N1326D, ΔAsn-1326, and I1405E/L1406F, we tested the alternative channel activators NAD and H\textsubscript{2}O\textsubscript{2}. TRPM2 variants showing ADPR gating also developed currents in the presence of NAD. The mutants N1326D, ΔAsn-1326, and I1405E/L1406F failed to respond to ADPR as well as to NAD (data not shown). Therefore, ADPR-dependent gating and NAD-dependent gating were indistinguishable in our expression system. We think that TRPM2 channel activation by NAD cannot be clearly separated from that by ADPR so far, because it cannot be excluded that NAD at the required high concentrations is contaminated by intracellularly interconverted to ADPR (19, 20).

Unfortunately, we were not consistently able to evoke the characteristic currents of wild-type and mutant TRPM2 channels by the application of H\textsubscript{2}O\textsubscript{2}. TRPM2 activation by oxidative stress appears to represent a cell-specific process not only in heterologous expression models but also in cells with endogenous expression of TRPM2. For example, TRPM2 is stimulated in cell lines derived from pancreatic β cells by H\textsubscript{2}O\textsubscript{2} (21) but not in neutrophil granulocytes (17).

In conclusion, the present study identified the highly conserved Asn-1326 as essential for ADPR gating of TRPM2. Furthermore, the data suggest that the ΔC-stretch of TRPM2 represents a structural element stabilizing the interactions of N1326 (and probably further residues immediately downstream) with other parts of the channel protein. Although the relevance of any ADPRase activity of TRPM2 remains unclear, our data demonstrated that the restitution of two amino acids that presumably enhance the enzymatic activity abolished the ADPR gating of TRPM2.

Acknowledgments—We thank Ilina Ionescu and Eberhard Jünghling for expert technical assistance.

REFERENCES

1. Clapham, D. E., Runnels, L. W., and Strubing, C. (2001) Nat. Rev. Neurosci. 2, 387–396
2. Minke, B., and Cook, B. (2002) Physiol. Rev. 82, 429–472
3. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) Cell 106, 559–598
4. Zitt, C., Halaszovich, C. R., and Luckhoff, A. (2002) Progr. Neurobiol. 66, 243–264
5. Birnbaumer, L., Yidirim, E., and Abramowitz, J. (2003) Cell Calcium 33, 419–432
6. Clapham, D. E. (2003) Nature 426, 517–524
7. Perraud, A.-L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P., and Scharenberg, A. M. (2001) Nature 411, 585–599
8. Sano, Y., Inamura, K., Miyake, A., Morishita, S., Yokoi, H., Matsushime, H., and Furuchi, K. (2001) Science 293, 1327–1330
9. Bessman, M. J., Frick, D. N., and O’Handley, S. F. (1996) J. Biol. Chem. 271, 25059–25062
10. Dunn, C. A., O’Handley, S. F., Frick, D. N., and Bessman, M. J. (1999) J. Biol. Chem. 274, 32318–32324
11. Gabelli, S. B., Bianchet, M. A., Bessman M. J., and Amzel L. M. (2001) Nat. Struct. Biol. 8, 467–472
12. Shen, B. W., Perraud, A.-L., Scharenberg, A., and Stoddard, B. L. (2003) J. Mol. Biol. 325, 385–398
13. Perraud, A.-L., Schmitz, C., and Scharenberg, A. M. (2003) Cell Calcium 33, 519–531
14. Perraud, A.-L., Shen, B., Dunn, C. A., Rippel, K., Smith, M. K., Bessman, M. J., Stoddard, B. L., and Scharenberg, A. M. (2003) J. Biol. Chem. 278, 1794–1801
15. Wehage, E., Eifeld, J., Heiner, I., Jungling, E., Zitt, C., and Luckhoff, A. (2002) J. Biol. Chem. 277, 23150–23156
16. Hara, Y., Wakamori, M., Ishii, M., Maseno, E., Nakada, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002) Mol. Cell 9, 163–173
17. Heiner, I., Eifeld, J., Halaszovich, C. R., Wehage, E., Jungling, E., Zitt, C., and Luckhoff, A. (2003) Biochim. Biophys. Acta 1631, 1045–1053
18. McHugh, D., Flemming, R., Xu, S.-Z., Perraud, A.-L., and Beech, D. J. (2003) J. Biol. Chem. 278, 1092–11006
19. Guse, A. H. (2002)Curr. Mol. Med. 2, 272–283
20. Guse, A. H. (2004) Curr. Mol. Med. 11, 847–855
21. Inamura, K., Sano, S., Morishita, H., Yokoi, A., Miyake, K., Nozawa, C., Kitada, H., Matsushime, K., and Furuchi, K. (2003) J. Membr. Biol. 191, 201–207
Sites of the NUDT9-H Domain Critical for ADP-ribose Activation of the Cation Channel TRPM2
Frank J. P. Kühn and Andreas Lückhoff

J. Biol. Chem. 2004, 279:46431-46437.
doi: 10.1074/jbc.M407263200 originally published online September 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407263200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 21 references, 5 of which can be accessed free at http://www.jbc.org/content/279/45/46431.full.html#ref-list-1