The Chlamydia protein CADD (Chlamydia protein associating with death domains) has been implicated in the modulation of host cell apoptosis via binding to the death domains of tumor necrosis factor family receptors. Transfection of CADD into mammalian cells induces apoptosis. Here we present the CADD crystal structure, which reveals a dimer of seven-helix bundles. Each bundle contains a diiron center adjacent to an internal cavity, forming an active site similar to that of methane monoxygenase hydrolase. We further show that CADD mutants lacking critical metal-coordinating residues are substantially less effective in inducing apoptosis but retain their ability to bind to death domains. We conclude that CADD is a novel redox protein toxin unique to Chlamydia species and propose that both its redox activity and death domain binding ability are required for its biological activity.

Chlamydiae are obligate intracellular bacteria and the causative agents of important sexually transmitted and disabling ocular (blinding trachoma) human diseases (1). Chlamydia engages in a unique relationship with its host. Upon entering host cells, the parasite starts a biphasic developmental cycle from the infectious form, called an elementary body, to a non-infectious, vegetative growth form, called a reticulate body, and then eventually back to the replication-incompetent infectious form (2). After the transition back to the infectious form, the host cell dies and releases its infectious load (3). To accommodate its life cycle, Chlamydia may inhibit apoptosis during the early stages of infection (4, 5) and promote apoptosis at later stages (6, 7).

Recently, the Chlamydia protein CADD has been shown to associate with tumor necrosis factor family proteins and to induce apoptosis when transfected into a variety of mammalian cell lines (8). CADD has no close homologues but does show 18% sequence identity with coenzyme PQP (pyrrolo-quinoline-quinone) (9). However, homologues of other members of the pathway are not found in Chlamydia species for which genome information is available. Indeed, ectopic expression of PqqC from Klebsiella pneumoniae failed to cause apoptosis, demonstrating the specificity of CADD-induced cell death (8). CADD is expressed late in the infectious cycle of Chlamydia trachomatis and is secreted into the host cytoplasm, where it co-localizes with tumor necrosis factor receptors in the proximity of the inclusion body. Sequence comparisons had suggested that CADD contains a death domain.

Here we present the crystal structure of CADD, which reveals an iron-containing redox enzyme that bears no resemblance to death domains. Mutagenesis of the active site of CADD reduced but did not eliminate its apoptotic activity, suggesting that both its catalytic activity and death domain binding activities contribute to its biological activity.

EXPERIMENTAL PROCEDURES

Mutation, Expression, and Purification of CADD—The open reading frame encoding CADD, CT610 (GenBank GI:3329055) from C. trachomatis was subcloned into pCDNA3-hemagglutinin (Invitrogen), pGEX-4T (Amersham Biosciences), pet21d (Invitrogen), and PEGFP-C2. The following mutations, Y170F (CADD-mut1) and E81A/H88A/Y170F/H174A (CADD-mut2) were introduced using the QuikChange kit (Stratagene), confirmed by DNA sequencing, subcloned into pet21d (Invitrogen), pGEX-4T, PEGFP-C2 and pDS-RED-C2, and transformed into Escherichia coli XLBlue. Glutathione S-transferase (GST) fusion proteins were obtained by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C for 8 h and then purified using glutathione-Sepharose (Amersham Biosciences). After thrombin cleavage, CADD was further purified on an s200 gel filtration column (Aekta-FPLC, Amersham Biosciences), concentrated to 12 mg/ml (AMICON), and flash-frozen in liquid nitrogen for long term storage at −80 °C. The selenomethionine-substituted protein was expressed as described (10) and purified as for the wild type, except that 5 mM tris(2-carboxyethyl)phosphine was added to the dialysis and gel filtration buffers.

Crystallography—Purified CADD was crystallized by the vapor diffusion method at room temperature using a sparse matrix screen (Hampton). Siting and hanging drops consisting of 3 μl of precipitant solution (10% (v/v) polyethylene glycol 12000, 20 mM cacodylate, pH 6.5) and 3 μl of protein solution (12 mg/ml protein) yielded crystals within 3–5 days. Crystals grew as very thin plates with dimensions of 200 × 200 × 20 μm in space group C2221. The crystal structure was determined by a selenomethionine-substituted protein (10). For data collection, crystals were transferred into cryobuffer (crystallization buffer with 25% (v/v) glycerol) and flash-cooled in liquid nitrogen.

Data Collection, Structure Solution, and Refinement—The three-dimensional MAD data set was collected from one single crystal, using synchrotron radiation at beamline X12B of the National Synchrotron Light Source. The data set was processed and scaled with the XDS program. Data were integrated using DENZO. The solution was found using the program SHELXS-97. Refinement was performed using SHELXL-97. The data set and the structure solution were confirmed by D*TAR (Kleyweg, W. E., and Terwilliger, T. C., unpublished data).

CADD was crystallized by the vapor diffusion method at room temperature using a sparse matrix screen (Hampton). Siting and hanging drops consisting of 3 μl of precipitant solution (10% (v/v) polyethylene glycol 12000, 20 mM cacodylate, pH 6.5) and 3 μl of protein solution (12 mg/ml protein) yielded crystals within 3–5 days. Crystals grew as very thin plates with dimensions of 200 × 200 × 20 μm in space group C2221. The crystal structure was determined by a selenomethionine-substituted protein (10). For data collection, crystals were transferred into cryobuffer (crystallization buffer with 25% (v/v) glycerol) and flash-cooled in liquid nitrogen.

Data Collection, Structure Solution, and Refinement—The three-dimensional MAD data set was collected from one single crystal, using synchrotron radiation at beamline X12B of the National Synchrotron Light Source. The data set and the structure solution were confirmed by D*TAR (Kleyweg, W. E., and Terwilliger, T. C., unpublished data).

CADD was crystallized by the vapor diffusion method at room temperature using a sparse matrix screen (Hampton). Siting and hanging drops consisting of 3 μl of precipitant solution (10% (v/v) polyethylene glycol 12000, 20 mM cacodylate, pH 6.5) and 3 μl of protein solution (12 mg/ml protein) yielded crystals within 3–5 days. Crystals grew as very thin plates with dimensions of 200 × 200 × 20 μm in space group C2221. The crystal structure was determined by a selenomethionine-substituted protein (10). For data collection, crystals were transferred into cryobuffer (crystallization buffer with 25% (v/v) glycerol) and flash-cooled in liquid nitrogen.
Table I
Crystallographic statistics of CADD

|                  | Native | Se-λ1 | Se-λ2 | Se-λ3 |
|------------------|--------|-------|-------|-------|
| Data collection  |        |       |       |       |
| Space group      | C222   | C222  | C222  | C222  |
| Cell dimensions (Å) | 77.55  | 77.63 | 77.62 | 77.51 |
| a                | 192.97 | 193.33| 193.38| 193.70|
| b                | 93.74  | 93.99 | 93.97 | 94.11 |
| c                |        |       |       |       |
| National Synchrotron Light Source beamline | X9B | X12B | X12B | X12B |
| Wavelength (Å)   | 0.954  | 0.973 | 0.9785| 0.961 |
| Resolution (Å)   | 95–2.5 | 30–3.1| 30–3.1| 30–3.1|
| Reflections (observed) | 98,532| 95,734| 88,847| 93,278|
| Reflections (unique) | 24,069| 13,117| 13,079| 13,212|
| Completeness (%) | 96.8 (94.2)| 99.8 (100.0)| 99.7 (100.0)| 99.7 (99.8)|
| Rmerge (%)       | 11.2 (2.3)| 7.4 (1.4)| 7.4 (1.5)| 6.3 (1.2)|

* Rmerge = Σ|I − I̅|/ΣI, where I is the observed intensity and I̅ is the average intensity from multiple observations of symmetry-related reflections, values in parentheses correspond to the highest resolution shell.

* Rcryst = Σ|Fobs − |Fcal|/Σ|Fobs|.

* Rfree = same as Rcryst but comprises a test set (5% of total reflections), which was not used in model refinement.

Light Source. Oscillation data were recorded in frames of 1° through a continuous angular range of 120° for the peak (λ = 0.9791 Å), the high energy remote (λ = 0.925 Å), and the inflection point (λ = 0.9794 Å). The native data set was collected at beamline X9B of National Synchrotron Light Source. All data were processed with the programs DENZO and SCALEPACK (11). The CADD structure was phased and traced using the program SOLVE/RESOLVE (12). Model building and refinement were carried out in O (13) and REFMAC5 (14). The final CADD model comprises three protein monomers (residues A7–A219, B1–B219, C7–C219), 6 Fe2+ ions with 3 closely bound putative water molecules, and 176 water molecules. Residues 1–6 and 220–231 were not visible in the electron density maps and therefore were not included in the model. Statistics for data collection, refinement, and model quality are summarized in Table I. Surface calculations were carried out with the CASTP server (15) and the protein-protein-interaction server (16). Figures were drawn with SPOCK (17) and PYMOL (DeLano Scientific LLC).

**Cell Culture, Transfections, and Apoptosis Measurements**—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Irvine Scientific) and supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics. Cells (106) were transfected with PEGFP-C2 plasmids containing CADDwt, CADD-mut1, and CADD-mut2, using LipofectAMINE (Invitrogen) following the vendor’s protocol. Both floating and adherent cells were recovered 1 day later and pooled, and the percentage of transfected (green fluorescent) cells with nuclear apoptotic morphology was determined by staining with 0.1 μg/ml 4’,6-diamidino-2-phenylindole (mean ± S.D.; n = 3). Cytosolic extracts from HeLa cells were subjected to immunoblotting and probed with rabbit polyclonal anti-green fluorescent protein (GFP) antibody (Invitrogen) for the presence of GFP-CADD fusion proteins.

**Protein Binding Assays**—A plasmid containing DR5 was in vitro transcribed and translated in the presence of [35S]methionine using the TnT kit from Promega. GST-CADD, GST-CADD-mut1 (data not shown), GST-CADD-mut2, and control GST-CD40 (cytosolic domain) fusion proteins were immobilized on glutathione-Sepharose at 1 μg/ml and incubated with in vitro translated target proteins for 2 h at 4 °C. Beads were then washed four times in 1 ml of 140 mM KCl, 20 mM Hepes, pH 7.5, 5 mM MgCl2, 2 mM EGTA, 0.5% Nonidet P-40, and analyzed by SDS-PAGE/fluorography.

**Mass Spectrometry and ICP-AAS**—Matrix-assisted laser desorption/ionization-time of flight, peptide mapping, and ICP-AAS-spectrometric analysis on the purified CADD protein were accomplished using standard techniques at the Facility for Mass Spectrometry at the Scripps Research Institute in La Jolla.

**RESULTS**

**CADD Structure**—Recombinant CADD from *C. trachomatis* was expressed in *E. coli*, purified, and crystallized. The crystal structure was determined by a selenium MAD experiment (10). CADD is a 231-residue protein, molecular mass = 26,734 Da, which forms a homo-dimer in solution, as judged by gel filtration. The CADD monomer is cylindrical with approximate dimensions of 45 × 29 × 37 Å. CADD folds into a seven-helix...
mostly parallel/anti-parallel bundle, where six α-helices (H1, H2, H3, H4, H5, H7) partly embrace the seventh helix (H6) (see Fig. 2A). According to the Structural Classification of Proteins Data Base (18), CADD belongs to the “heme-oxygenase” fold. Helices H1, H3, H4 are kinked and can therefore be represented as separate shorter α-helices denoted as A and B. This is especially true for helix H3, where a hairpin loop, residues 82–87, is inserted (Figs. 1A and 2).

The CADD dimer is formed through an interaction via helices H2 and H3A, residues 59–85 (Figs. 1B and 2). The interface-accessible surface area is 915 Å²/monomer, which accounts for 9.2% of the accessible surface area of the CADD dimer. The interaction is predominantly hydrophobic (55% non-polar atoms) but also includes a number of polar interactions and salt bridges. The most similar structures found using the DALI server (19) are: PqqC (20), with an r.m.s.d. of 2.9 Å for the superposition of 221 Co atoms and 18% sequence identity; human heme-oxygenase (21), with an r.m.s.d. of 2.9 Å for 199 Co atoms and 11% sequence identity; the R2 subunit of ribonucleotide reductase (R2-RNR) (22), with an r.m.s.d. of 3.2 Å for 178 Co atoms and 12% sequence identity; and the α-subunit of methane monoxygenase (MMOH) (23), with an r.m.s.d. of 3.1 Å for 174 residues and 9% sequence identity. Although none of the active sites are conserved, each of these enzymes appears to be a reox enzyme, suggesting that this fold is particularly suitable for this type of enzyme. According to sequence similarity searches with the bioinformatics server Fold and Function Assignment System (24), CADD shares distant sequence homology with transcription enhancement gene A transcription factors (25) and can be used as a template to obtain homology models for these proteins.

The Active Site—The seven helices of CADD provide the scaffold for a narrow internal cavity equipped with a di-metal center (Figs. 2 and 3A). The experimental electron density map clearly indicates the presence of two metal ions coordinated by 6 residues (Glu-81, His-88, Glu-142, His-174, Asp-178, His-181) (Figs. 2 and 3A). The di-metal site is located in the center of the molecule adjacent to the cavity, which most likely serves as the active site. Atomic absorption measurements using ICP-AAS revealed the presence of iron and small but significant amounts of zinc in the protein. This indicates the presence of a di-iron site, which, judged by difference maps and elevated B-factors, is not fully occupied in the CADD crystals. The small amounts of zinc might be due to oxidation and partial replacement of iron for zinc, which has been observed in several crystal structures of di-iron-containing proteins (26). The di-metal center appears to be octahedrally coordinated and bridged by a glutamate residue (Glu-81) and a water molecule or hydroxide ion. Fe1 is coordinated by two histidines (His-174, His-88) and the glutamate (Glu-81), as well as the putative tyrosyl radical carrying Tyr-170 and the putative OH⁻ molecule are depicted in ball and stick format. The 2Fe¹−Fe² electron density map is contoured at 1.5 σ. B, close-up view of the CADD molecule in a transparent surface representation (orange) showing the internal cavities, the di-metal site (purple spheres), and surrounding residues in ball and stick format. The two possible entrances are located on the right side of the metal site (E1) next to loop L3 and on the bottom of the molecule (E2) close to helix H1b-H6.

Fig. 2. Multiple sequence alignment of CADD proteins from different bacterial sources. Shown are sequences of CADD from C. trachomatis (C_tru, NP_220127.1; GI:15605341) Chlamydia muridarum (C_mur, NP_297273.1; GI:15835514), Chlamydia pneumoniae J138 (C_pne, NP_300818.1; GI:15836294), and PqqC Eubacterium K. pneumoniae (P27505 GI:130800). Helices and loops as observed in the structure of CADD are indicated above its sequence. Regions participating in the dimer interface are underlined. Metal-coordinating residues are bold, and residues lining the internal cavity are highlighted in gray. For comparison, the PqqC active site residues are shown in white letters with a black background.

Fig. 3. Active site analysis. A, the di-iron site. The metal-coordinating active site residues Glu-81, His-88, Glu-142, His-174, Asp-178, and His-181 as well as the putative tyrosyl radical carrying Tyr-170 and the putative OH⁻ molecule are depicted in ball and stick format. The 2Fe¹−Fe² electron density map is contoured at 1.5 σ. B, close-up view of the CADD molecule in a transparent surface representation (orange) showing the internal cavities, the di-metal site (purple spheres), and surrounding residues in ball and stick format. The two possible entrances are located on the right side of the metal site (E1) next to loop L3 and on the bottom of the molecule (E2) close to helix H1b-H6.
core is largely aromatic and also contains a buried lysine (Lys-152). A system of cavities spans across the core of the molecule, with two potential openings next to loop L3 and between helices H1B and H5. One opening, E1, penetrates the surface of the protein between helices H2, H3, and the unique loop L3 (Figs. 1A and 3B). It is lined by residues Ile-51, Pro-55, Ile-89, and Glu-82. An alternative access path, E2, leads from the di-iron site through a narrow opening into a second cavity lined by residues Met-21, Tyr-43, Tyr-47, Trp-92, Ile-148, Ala-149, Phe-171, Ala-149, Lys-152, and Tyr-27 and from there to the surface next to residues Trp-30 and Asp-151 (helices H1B and H5). The size of the active site cavity openings restricts the substrates to small compounds such as O2, H2O2, CH4, CH3OH, CO, or CO2. Larger molecules could only pass through by means of a conformational change.

The active site of CADD is similar to that found in RNR-R2 from E. coli (Protein Data Bank accession code 1xsm). The helices forming the core that contains the active site can be superimposed with an r.m.s.d. of 2.8 Å. The function of RNR-R2 is to generate a tyrosyl radical on an adjacent tyrosine with the help of its di-iron center. The organic free radical is transferred to the RNR-R1 subunit, which catalyzes the de novo production of deoxy nucleotides (22). Interestingly, CADD also contains a tyrosine (Tyr-170) next to the di-iron center. These similarities raise the question whether the physiological function of CADD is the production of radicals for RNR-R1. However, no equivalents are seen for Asp-84, Asp-237, and Trp-48, which are critical residues for the radical initiation pathway proposed in RNR-R2 (Tyr-122-Asp-84-Fe1-His-118-Asp-237 to Trp-48) (26). Taken together, these findings indicate that CADD cannot function as a RNR-R2 but might use a tyrosyl-radical for catalysis.

Cellular Activity of CADD—To test whether Tyr-170 and the di-metal site are involved in the toxicity of CADD, we generated two active site mutants by PCR mutagenesis and tested their apoptotic activity through transfection experiments in mammalian cells. The role of Tyr-170 was tested with a Y170F (CADD-mut1) mutant. To prevent the formation of a functional di-metal center, we made the quadruple mutant of the metal-coordinating residues: E81A/H88A/H174A/Y170F (CADD-mut2). When equivalents of each plasmid DNA were transfected into HeLa cells, CADD-mut1 showed a decrease in toxicity of about 5–15% when compared with the wild-type. CADD-mut2 showed more than 60% reduction in apoptotic activity (Fig. 5A). Immunoblotting shows (Fig. 5B) that both CADD mutants are expressed at similar or higher levels to wild-type. This indicates that the mutants, especially CADD-mut2, are better tolerated by the transfected mammalian cells than the wild-type. To address the question of whether the active site mutant proteins still bind to death receptors, we carried out an in vitro DR5 binding assay (Fig. 5C), comparing GST-CADD-wt, GST-CADD-mut1 (data not shown), and GST-CADD-mut2. CADD wild type and active site mutants show comparable binding to death receptor DR5, indicating that the active site mutations do not alter the DR5 binding activity of CADD.

DISCUSSION

The crystal structure shows that CADD shares similarity to heme-oxygenase and PqqC enzymes. The sequence similarity and “PqqC-like” annotation for CADD proteins are reflected by the same fold, but the active sites are not conserved, and the two proteins are therefore functionally and most likely also evolutionarily unrelated (20). CADD is consequently an orphan unique to Chlamydia species, which further emphasizes its role as a highly specific toxin that evolved in this intracellular parasite. Comparison with the more distant structural homologues, RNR-R2 (22) and MMOH (23), reveals di-iron active sites in a strikingly similar structural context. Although the three proteins belong to different fold subclasses (CADD shows the heme-oxygenase fold, whereas RNR-R2 and MMOH belong to the ferredoxin fold), the helices forming the core containing the active site can be superimposed with an r.m.s.d. <2.8 Å. The active site of CADD is structurally similar to that in
A structural comparison with the di-iron center in MMOH from Methylococcus capsulatus (Protein Data Bank accession code 1mhyD) (23) reveals strong conservation of the metal-coordinating residues, except for a difference in the coordination of Fe1 in CADD, where Glu-114 is replaced on the other side of Fe1 with His-174 (Fig. 4, A and B). A detailed analysis of the active sites further reveals that in contrast to RNR-R2, MMOH and CADD contain an internal cavity next to the di-iron center (Fig. 4C). In MMOH, the cavity functions as the site of catalysis, where substrate and product access the di-iron center through the tunnel-like cavity from the bottom of the molecule. CADD contains a similar tunnel when the entrance next to Trp-92, between H1B and H5, is used (Fig. 3B). On the other hand, the opening next to the loop L3 is a potential region for a conformational change that could open the cavity to the outside for the exchange of substrate and product. Thus, CADD is most likely an enzyme similar to MMOH (23), which uses an internal active site equipped with a di-iron center to catalyze redox reactions on small molecule substrates. Further biochemical studies are needed to determine the reaction catalyzed by CADD.

Transfection assays with a CADD mutant lacking critical metal-coordinating residues establish a direct connection between the di-iron site and the apoptotic activity of CADD. Alterations at the active site, which is buried within the molecule, do not abolish interaction with death receptors, which suggests that the optimal interaction of apoptosis by CADD requires both the intracytoplasmic cross-linking of death receptors as well as its redox activity.

Acknowledgments—We thank Jose Maria de Pereda for valuable discussions and helpful suggestions.

REFERENCES
1. Antilla, T. (2001) J. Am. Med. Assoc. 285
2. Schachter, J. (1988) Curr. Top. Microbiol. Immunol. 138, 109–139
3. Belland, R. J., Siddiqui, M. A., Crane, D. D., Hogan, D. M., Whitmire, W., McClarty, G., and Caldwell, H. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13984–13989
4. Fischer, S. F., Schwarz, C., Vier, J., and Hacker, G. (2001) Infect. Immun. 69, 7121–7129
5. Geng, Y., Shane, R. B., Berencsi, K., Gonzalez, E., Zaki, M. H., Margolis, D. J., Trinchieri, G., and Roel, A. H. (2000) J. Immunol. 164, 5522–5529
6. Schoier, J., Ollinger, K., Kvatavro, M., Soderlund, G., and Kihlstom, E. (2001) Microb. Pathog. 31, 173–184
7. Perfettini, R., Hospital, V., Liao, J., and Reed, J. C. (2002) J. Biol. Chem. 277, 9833–9836
8. Meulenber, J. C., Sellin, J., and Postma, P. W. (1990) FEMS Microbiol. Lett. 59, 337–343
9. Harrison, C. J., Bohm, A. A., and Nelson, H. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 101, 7121–7129
10. Harrison, C. J., Bohm, A. A., and Nelson, H. C. (1994) Science 263, 224–227
11. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 267, 307–326
12. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 849–861
13. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
14. CCP4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
15. Geng, Y., Shane, R. B., Berencsi, K., Gonczol, E., Zaki, M. H., Margolis, D. J., Trinchieri, G., and Roel, A. H. (2000) J. Immunol. 164, 5522–5529
16. Schoier, J., Ollinger, K., Kvatavro, M., Soderlund, G., and Kihlstom, E. (2001) Microb. Pathog. 31, 173–184
17. Perfettini, R., Hospital, V., Liao, J., and Reed, J. C. (2002) J. Biol. Chem. 277, 9833–9836
18. Meulenber, J. C., Sellin, J., and Postma, P. W. (1990) FEMS Microbiol. Lett. 59, 337–343
19. Harrison, C. J., Bohm, A. A., and Nelson, H. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 101, 7121–7129
20. Harrison, C. J., Bohm, A. A., and Nelson, H. C. (1994) Science 263, 224–227
21. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 267, 307–326
22. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 849–861
Structure of the *Chlamydia* Protein CADD Reveals a Redox Enzyme That Modulates Host Cell Apoptosis

Robert Schwarzenbacher, Frank Stenner-Liewen, Heike Liewen, Howard Robinson, Hua Yuan, Ella Bossy-Wetzel, John C. Reed and Robert C. Liddington

*J. Biol. Chem.* 2004, 279:29320-29324.  
doi: 10.1074/jbc.M401268200 originally published online April 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401268200

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 23 references, 8 of which can be accessed free at http://www.jbc.org/content/279/28/29320.full.html#ref-list-1