Identification of Caspase-1 and Caspase-3 Substrates And Study on Caspase-1 Substrates in Glycolytic Pathway

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ABSTRACT

Apoptosis is executed by caspase-mediated cleavage of various proteins. Elucidating the consequence of substrate cleavage provides us with insight into cell death and other biological processes. In this study, we applied the diagonal gel approach, a proteomic strategy, to identify substrates of the inflammatory caspase, caspase-1 and the cell death executioner caspase, caspase-3. Our results showed significant overlap between the substrates cleaved by both caspase-1 and –3. Such substrates are implicated in common cellular functions, including maintenance of the cytoskeleton, folding of proteins, translation, glycolysis, bioenergetics, signaling and trafficking. An important finding is that many glycolysis enzymes were targeted specifically by caspase-1. Processing of these glycolysis enzymes by caspase-1 was confirmed by cleaving *in vitro* transcribed and translated substrates with recombinant caspase-1. We have focused our further analysis on certain glycolysis enzymes. We have characterized the caspase-1 cleavage site in GAPDH. Point mutation of the Aspartic acid at position 189 to Alanine (D189A) in GAPDH blocked its cleavage by caspase-1. *In vivo*, in a mice model of septic shock, characterized by hyperactivation of caspase-1, we observed depletion of the full-length forms of these glycolysis enzymes in the diaphragm muscle. Further studies in caspase-1 deficient mice will confirm whether this depletion, in caspase-1 proficient mice, was due to caspase-1 processing of the glycolysis enzymes. This provides a direct link between caspase-1 activation and inhibition of glycolysis, which might have important implications on loss of muscle contractility in septic shock.

RÉSUMÉ

L'apoptose ou la mort cellulaire programme est un phénomène medié en partie par les caspases capables de cliver plusieurs substrats. Élucider le rôle et les conséquences du clivage de ses différents substrats nous fournira plus éléments dans la compréhension de la mort cellulaire et autres processus biologiques. Dans cette étude, nous avons appliqué l'approche du gel diagonale, une stratégie proteomic, afin d'identifier tous les substrats clives par la caspase inflammatoire, caspase-1, et les comparés aux substrats clives par la caspase apoptotique, caspase-3. Nos résultats montrent dans un premier temps, qu'un nombre considérable de substrats est clivé a la fois par caspase-1 et aussi par caspase-3, cela appuie leur commun dans des fonctions cellulaires, y compris l'entretien du cytosquelette, conformation protéique, la traduction, la glycolyse, bioénergie, la signalisation et le trafique cellulaire. Dans notre approche, nous avons remarque que plusieurs enzymes impliquées dans la glycolyse sont des substrats uniques de caspase-1. Le clivage de ces enzymes de glycolyse par caspase-1 a été confirmé *in vitro* en présence de la protéine recombinante caspase-1. Nous avons concentré notre analyse supplémentaire sur certaines enzymes telle que GAPDH. Nous avons pu caractérisé le site de clivage de la GAPDH par capase-1, et en réalisant une mutation cible de l'acide Aspartique de la position 189 en Alanine (D189A), on aboutit a un blocage du clivage par caspase-1. Nos résultats *in vitro* ont été complétés par une approche *in vivo* en utilisant un model animal de choc septique, une souris caractérisé par une hyperactivation de caspase-1, nous avons pu observé une décroissance dans les formes longues de ces enzymes dans le muscle du diaphragme. L'utilisation de souris défectueuses en caspase-1 vont nous démontrer si le clivage et la diminution dans la forme longue de ces enzymes est réellement le résultat d'un clivage par caspase-1. Cela fournira un lien direct entre l'activation de caspase-1 et l'inhibition de la glycolyse qui pourra avoir des percussions importantes sur la perte de contractilité du muscle dans le choc septique.

CONTRIBUTION OF AUTHORS

The research conducted and presented in this thesis is entirely my own except that septic shock mice induced with LPS was done by Dr. Sabah Hussain's lab. Dr. Maya Saleh supervised the majority of the work. This thesis was written by me with correction by Dr. Maya Saleh.

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ABREVIATIONS

APAF-1	apoptosis protease-activating factor-1
APP	amyloid β precursor protein
ASC	apoptosis-associated speckle-like containing protein
ATCC	American type culture collection
ATP	adenosine triphosphate
BOC	benzyloxycarbonyl
cAMP	cyclic adenosine monophosphate
CARD	caspase recruitment domain
CrmA	cytokine response modifier A
CTL	cytotoxic T lymphocyte
DD	death domain
DED	death effector domain
DIAP	*Drosophila* inhibitor of caspase
DISC	death-inducing signaling complex
DR	death receptor
DTT	dithiothreitol
EPO	erythropoitin
FACs	fluorescent-activated cell sorting
FADD	Fas-associated protein with a death domain
FBS	fatal bovine serum
FLICA	fluorochrome inhibitor of caspases
FMK	fluorometyl ketone
GAPDH	glyceraldehydes-3-phosphate dehydroenase
GLUT1	glucose transporter 1
HIF	hypoxia-inducible factor

HSP	heat shock protein
IκB	Inhibitor of NF-κB
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated Dnase
IL	interleukin
IPAF	ICE protease-activating factor
ITT	*in vitro* transcription and translation
LB	Luria-Bertani
LC-MS	liquid chromatography-fed mass spectrometry
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	Leucine rich repeats
MAPK	mitogen-activated protein kinase
MBP-1	*Myc* promoter-binding protein-1
moi	multiplicity of infection
MOMP	mitochondrial outer membtane permeablilization
NAIP	neuronal apoptosis inhibitor protein
NF-κB	nuclear Factor kappa B
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PMA	phorbol-12-myristate-13-acetate
PYD	Pyrin domain
Rb	retinoblastoma-associated proteein
RBC	red blood cells
RIP	receptor interacting protein

SDS	Sodium dodecyl sulfate
SREBP	sterol regulatory element binding proteins
TBS	tris-buffered saline
TIM	triosephosphate isomerase
TNF	tumor necrosis factor
TNF-R1	TNF receptor 1
TRADD	TNFR-associated death domain
TRAF1	TNF receptor-associated factor-1
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-chromasome-linked inhibitor of apoptosis protein

LITERATURE REVIEW:

Apoptosis

Apoptosis is a genetically programmed cell death. This process can be triggered by a variety of stimuli, including cytokines, hormones, viruses, and toxic insults (Creagh et al., 2003). It is a fundamentally important process necessary for development and homeostasis of multicellular organisms. Inappropriate apoptosis causes human diseases, including neurodegenerative diseases, autoimmune disorders and cancer (Nicholson and Thornberry, 1997). Apoptosis is the major form of cell death, which is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing, all of which result in recognition and phagocytosis of apoptotic cells by phagocytes, thereby preventing an inflammatory response. This process is coordinated by a group of proteases, the caspases (Nicholson and Thornberry, 1997).

Caspase family

The name caspase (derived from cysteinyl-aspartate-specific proteinase) illustrates two distinguishing features of these enzymes: first, their catalysis is governed by a critical conserved cysteine residue; second, they specifically cleave after an Aspartate amino acid. To date, 14 mammalian family members have been identified. (Caspase-15 has been recently identified as an apoptotic caspase expressed in various mammalian species but not in humans (Eckhart et al., 2005)). 13 human caspases are known. A phylogenetic analysis classifies the family into two subfamilies: the caspase-1 subfamily (caspases-1, -4, -5, and -12) and the caspase-3 subfamily (caspases-2, 3, 6, 7, 8, 9, and 10). Caspase-14 is confined to the skin where it acts in skin differentiation. Caspase-1 subfamily members predominantly play a role in inflammation, while caspase-3 subfamily members are mainly involved in apoptosis (Nicholson, 1999).

The general structure of caspases

Caspases are synthesized as catalytically dormant proenzymes containing three domains: an N-terminal prodomain, a large subunit and a small subunit. They all have a conserved QACXG sequence in their catalytic center. In general, caspases can autocleave themselves or cleave other caspases. Based on their known or hypothetical roles in apoptosis, caspases are further divided into two functional subgroups: initiator and executioner caspases. Initiator caspases (caspases-1, 2, 8, 9 and 10) are responsible for initiating caspase-activation cascades. The initiator caspases have long prodomains containing caspase recruitment domains (CARDs) or death effector domains (DEDs), which promote caspase activation. The downstream or executioner caspases (caspases-3, 6, 7, and as we present in this thesis caspase-1) are responsible for the actual dismantling of the cell during apoptosis and pyroptosis, which is a caspase-1-induced cell death associated with inflammation. Executioner caspases usually have short prodomains.

Caspase activation

The initiator caspases exist as monomers in the cytosol, and are activated by oligomerization in macromolecular complexes assembled by scaffolding molecules. Oligomerization of initiator caspases is triggered by the assembly of platforms that recruit caspases into close proximity. When oligomerized within macromolecular structures such as the DISC (death-inducing signaling complex), the apoptosome or the inflammasome, initiator caspases form an active site and subsequent cleavage within the inter-domain linker stabilizes their oligomers (Boatright et al., 2003, Green, 2003 #25; Green, 2003). The executioner caspases exist as inactive proform dimers. Their activation occurs through cleavage by the initiator caspases (Green, 2003). The activation pathways of the apoptotic caspases are discussed below as the extrinsic, intrinsic and granzyme B activation pathways (Creagh et al., 2003). The activation of the inflammatory caspases is reviewed in the caspase-1 section.

The extrinsic pathway

This pathway is responsible for eliminating activated T cells (AICD-activation-induced cell death), as well as virally infected or transformed cells. The 3 molecular players that are engaged at the death-inducing signaling complex or DISC of the extrinsic pathways are: 1) Death receptors, 2) FADD, and 3) the initiator caspases – caspases-8 or –10. Death receptors include the TNF receptor 1 (TNF-R1), Fas also known as CD95, and the TRAIL receptors DR4 and DR5. FADD, also known as FAS-associated protein with a death domain, is a bimodular adaptor that contains a death domain DD but also a death effector domain DED. DDs, DEDs, CARDs (caspase recruitment domains) and pyrin domains PyDs belong to a group of structural domains known as the "death fold" domains that consist of 6 α-helices and that mediate

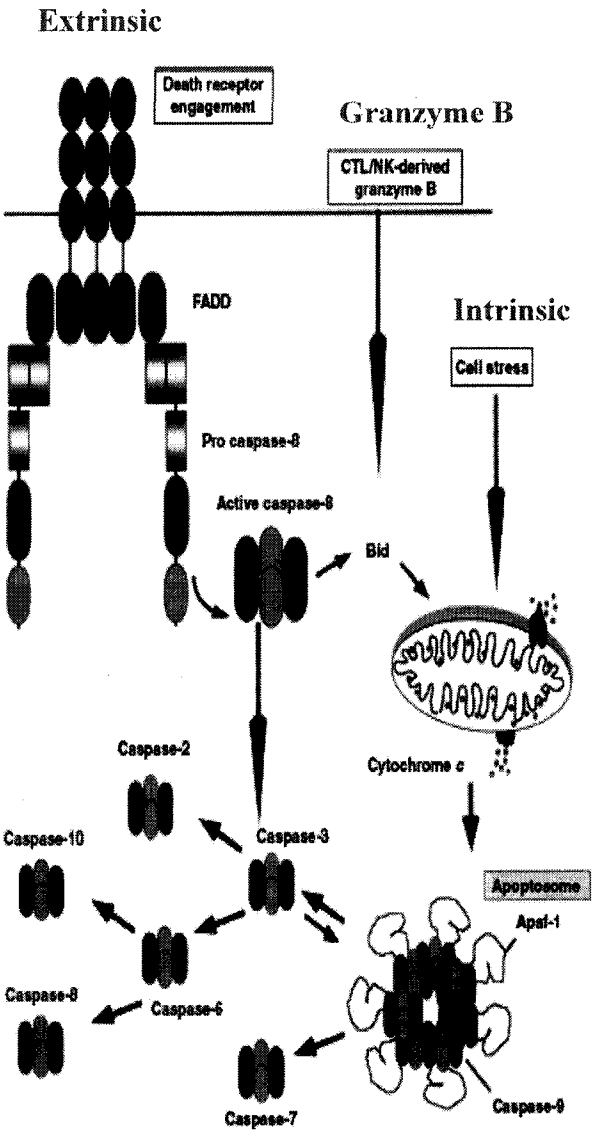


Figure 1 Apoptosis Pathways (Creagh, Conroy et al. 2003)

homotypic protein-protein interactions (e.g. DD-DD, DED-DED, CARD-CARD and PyD-PyD). Upon binding to their ligands, death receptors oligomerize on the cell surface and assemble the DISC. In their cytoplasmic tails, death receptors contain a death domain (DD). In response to ligand binding, the death receptor DD associates with the death domain of FADD, which in turn recruits Caspase-8 via its death effector domain (DED). This results in the activation of the initiator caspases-8 and 10. The activated initiator caspases can then cleave and activate executioner caspases (figure 1) (Earnshaw et al., 1999). TNFR1 binds to an additional adapter protein TRADD, which in turn binds to FADD. New evidence shows that the activation via TNF-R1 may happen in two steps: TNF-R1 associates with TRADD upon binding to its ligand, and then dissociates from TRADD due to TRADD undergoing some modification, possibly phosphorylation, which allows TRADD to recruit and activate caspase-8 within the cytosol (Micheau and Tschopp, 2003).

The intrinsic pathway

This pathway is responsible for the maintenance of normal cell number in neuronal development as well as apoptosis in response to environmental insults. Activation of this pathway is initiated by stimuli such as cytotoxic drugs, heat shock, ionizing radiation and other cellular stresses leading to mitochondrial outer membrane permeabilization (MOMP). MOMP results in cytochrome c release from the mitochondrial intermembrane space into the cytosol. Cytosolic cytochrome c binds to the WD40 repeat domain in APAF-1 (apoptosis protease-activating factor-1), which induces an ATP-mediated assembly of a heptamer complex and the recruitment of pro-caspase-9 via binding of the caspase-9 CARD to the CARD in APAF-1. This protein complex is known as the apoptosome. The activated caspase-9 then cleaves excutioner caspases (figure 1) (Creagh et al., 2003).

Granzyme B-initiated caspase-activation pathway

Granzyme B, a serine protease derived from cytotoxic T lymphocyte (CTL) and Natural Killer cells, shares with caspases a preference for aspartate residues in the P1 position of its substrates. It is the only protease other than caspases known to cleave caspases (Nicholson, 1999). It can cleave caspases-3 and –8 as well as the caspase-8 substrate Bid and initiates apoptosis, building up an alternative apoptosis pathway in virally infected and tumor cell (figure 1) (Atkinson et al., 1998; Medema et al., 1997)

Caspase activation networks

During apoptosis, initiator caspases are activated to cleave executioner caspases. Caspase activation in the extrinsic pathway proceeds from the death receptor induced caspase-8 cleaving of caspase-3 which cleaves caspase-6; the intrinsic pathway proceeds from the cytochrome c:Apaf-1 apoptosome complex by caspase-9 cleaving caspase-3 and caspases-6 and -7. At the same time, downstream caspases can cleave upstream caspases. For example, caspase-6 can cleave procaspases-8 and –10, and caspase-3 can cleave procaspase-9. The cleavage of upstream caspases by downstream caspases acts as a positive feedback mechanism to enhance apoptosis (Earnshaw et al., 1999).The protein Bid serves as a "cross-talking" molecule that transmits the apoptotic signal from the extrinsic to the intrinsic pathway. When caspase-8 is activated via the extrinsic pathway, it cleaves Bid into its active form, tBid (truncated Bid). tBid translocates to the mitochondria and activates pro-apoptotic members of the Bcl-2 family, Bax and Bak, to cause mitochondrial outer membrane permeabilization (MOMP). In addition, the mitochondrial proteins Smac/DIABLO and Omi/HtrA2 are released to prevent the inhibition of caspases mediated by the IAPs (inhibitors of apoptosis proteins). Granzyme B also cleaves Bid into tBid and feeds the apoptotic signal to the intrinsic pathway (Green, 2003)

Regulation of caspases in apoptosis

The activation of caspases is under the control of two protein families: 1) the pro- and anti-apoptotic proteins of the Bcl-2 family, and 2) the IAP (inhibitor of apoptosis protein) family.

The Bcl-2 family

The Bcl-2 family has anti- and pro-apoptotic members. During apoptosis, mitochondrial outer membrane permeabilization (MOMP) occurs. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-X_L, Bcl-w and Mcl-1) prevent MOMP, and thus prevent cytochrome c release and the consequent apoptosome assembly and caspase-9 activation. On the contrary, the pro-apoptotic members BH123 proteins (Bax, Bak and Bok), when activated, oppose the actions of the anti-apoptotic members and promote MOMP. The BH123 proteins are activated by another set of BH3-only proteins (Bim, Bid, Bad, Bmf, BNIP-3, Puma, Noxa) (Green, 2003)

The IAP family

Bcl-2 family members regulate caspases by acting upstream of the mitochondria in the intrinsic pathway. Downstream of the mitochondria, when procaspase-3 is cleaved by caspase-9, although enzymatically active at this point, caspase-3 is under a "brake" because it is bound by XIAP (X-chromosome-linked inhibitor of apoptosis protein), a member of the IAP family. Relief of inhibition of caspase-3 by XIAP is facilitated by competition of binding of Smac/DIABLO or Omi/Htra2 (released from mitochondria during MOMP) to XIAP (Du et al., 2000; Verhagen et al., 2000).

Caspase proteolytic properties

Caspases are cysteine proteases. They are distinct from other cysteine proteases owing to their absolute requirement of an Asp in the P1 position N-terminus of the scissile bond. Of the currently known human and mouse proteases, only Granzyme B shares this specificity (Nicholson, 1999). The residue after the scissile bond (P1') is also important in that charged or bulky residues are poorly tolerated; Gly, Ala, Thr, Ser and Asn allow efficient catalysis, but Glu, Asp, Lys, Arg, Trp do not (Stennicke et al., 2000). Substrate specificity is determined by the residue at the P4 position. Screening of a positional scanning combinatorial substrate library divided caspases into three groups. Group I caspases (1, 4, and 5) prefer bulky hydrophobic amino acids such as Tyr or Trp in the P4 position. Group II caspases (2, 3, and 7) require a P4 Asp. Group III caspases (6, 8, 9, and10) prefer branched chain aliphatic amino acids in the P4 position (Nicholson, 1999) (Figure 2).

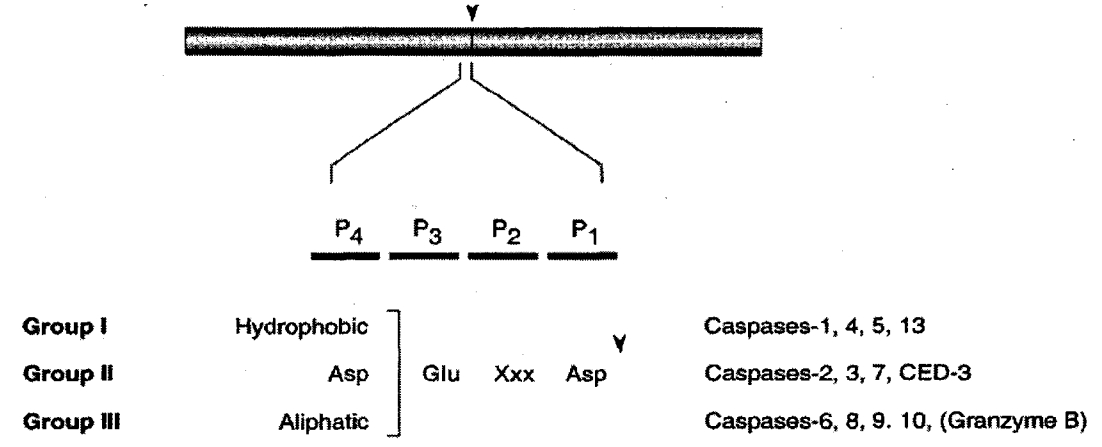


Figure 2 Caspase Substrates Specificity (Nicholson 1999)

For natural proteins, however, the presence of a preferred cleavage site consisting of four specific amino acids is not sufficient. Enzyme regions distant from the catalytic site that participate in substrate binding (exosites) have been implicated in the recognition of

natural substrates. One example is derived from the crystal structure of caspase-8 bound to its natural inhibitor p35, which is the only caspase structure solved with a natural substrate bound to the caspase. p35 contains a loop that interacts with a face of caspase-8 distant from its active site, suggesting that exosites interactions with the substrate might also be important (Fisher et al., 1999; Xu et al., 2001). The exosite interactions might explain the discrepancies in the cleavage consensuses between synthetic and natural substrates.

Caspase substrates in apoptosis

During apoptosis, caspases cleave a large number of proteins to disrupt cellular functions, which accounts for the phenotype seen in cells undergoing apoptosis. Caspases cleave key structural components of the cytoskeleton such as actin, fodrin, vimentin and keratin etc., which contributes to cell shrinkage and cell detachment, as well as interrupts antiapoptotic integrin signaling. Caspases cleave ICAD (inhibitor of caspase-activated Dnase). Cleavage of ICAD releases CAD liberating it to translocate to the nucleus where it fragments DNA. Caspases cleave numerous proteins involved in the transducation and amplification of apoptotic signals. For example, caspases target the survival NF-κB pathway, cleaving p65 and IκB-α, TRAF-1 and RIP-1. Caspases also cleave critical proteins involved in the regulation of the cell cycle, such as cyclin E, MDMX, Rb, causing cell cycle attenuation. Additionally, caspases cleave many proteins involved in the translation machinery, resulting in the blockade of protein translation (Fischer et al., 2003).

Caspase substrates involved in neuropathologies

The cleavage of specific substrates is directly linked to pathogenesis of certain neurodegenerative disorders. Huntingtin, which is mutated in Huntington's Disease and

possesses an expanded polyglutamine stretch in its N-terminus, is cleaved by caspase-3, and it is the aggregation of its N-terminal fragments in neurons which is suggested to cause the disease (Zhang et al., 2003b). APP, the amyloid β precursor protein involved in Alzheimer's disease, is additionally cleaved by caspase-3 at $VEVD^{664}$. The consequent generation of the cytosolic fragment (C31) is associated with cell toxicity. Transgenic mice expressing an uncleavable mutant of APP (mutation of the cleavage site to alanine $VEVD^{664} \downarrow A$) are protected from the disease (Galvan et al., 2006).

Synthetic inhibitors

The sufficiency of a P4-P1 four amino acid recognition motif for caspases serves as the basis of inhibitor design. Reversible inhibitors of caspases are generated by coupling caspase-specific peptides to certain aldehyde, nitrile or ketone compounds; irreversible inhibitors are generated by coupling caspase-specific peptides to fluoromethyl ketone (FMK). To enhance cellular permeability, the inhibitors are synthesized with a benzyloxycarbonyl group (also known as BOC or Z), and they are widely used in both *in vitro* cell culture and *in vivo* studies. Examples of commercially available inhibitors are z-YVAD-FMK for caspase-1; z-DEVD-FMK for caspase-3; z-IETD-FMK for caspase-8 and a pan-caspase inhibitor z-VAD-FMK (Nicholson, 1999).

Naturally occurring protein inhibitors

Cytokine response modifier A (CrmA) is a 38 KDa protein from cowpox virus, which facilitates viral infection through inhibition of cytokine production and apoptosis by efficient inhibition of both caspases-1 and –8 (Komiyama et al., 1994). p35 is another viral protein from baculovirus. Cleavage of p35 by a caspase results in the formation of a caspase-p35 complex. The presence of this complex prevents caspases from initiating the

apoptotic cascade (Xue and Horvitz, 1995).

Non-apoptotic functions of caspases

Caspases play an essential role in apoptosis. But recent studies have uncovered novel functions of caspases in non-apoptotic processes including the inflammatory response, immune cell proliferation, cell differentiation and cell migration. The major function of caspase-1 is in regulation of the inflammatory response by processing pro-IL-1β, pro-IL-18 and pro-IL-33 into their mature cytokine forms (Li et al., 1995; Schmitz et al., 2005). New evidence show that caspase-1 could also contribute to inflammation by the activation of the NF-κB pathway through the interaction between the CARD domain of caspase-1 and the kinase RIP1 (Lamkanfi et al., 2004). Caspase-3, the main death effector caspase, can process pro-IL-16 into its active form (Zhang et al., 1998), suggesting a role of caspase-3 in inflammation. Caspase-8 appears to play an essential role in T-cell proliferation and activation. It has been shown that caspase-8 cleaves the kinase Wee1 during T-cell proliferation (Alam et al., 1999), which prevents phosphorylation of the cell cycle-regulating kinase Cdc2 needed to promote cell cycle progression. Caspase-8 is also implicated in cell differentiation. Conditional knockout of caspase-8 in the T cell-lineage revealed a defect in T cell homeostasis and activation in the absence of caspase-8 (Salmena and Hakem, 2005). Additionally, conditional knock-out of caspase-8 in the myelomonocytic lineage resulted in an arrest in macrophage differentiation (Kang et al., 2004). Caspases are also involved in the differentiation process associated with enucleation of erythrocytes and keratinocytes (Zandy et al., 2005; Zermati et al., 2001). The enucleation process exhibits some similarities to apoptosis such as chromatin condensation and degradation of nuclear components (Morioka et al., 1998). Caspases are probably activated through a mitochondrial-dependent pathway and cleave proteins such as lamin and actin, which are responsible for nuclear disassembly and chromatin condensation (Zermati et al., 2001).

Although caspases are activated in those cell types during enuleation, the activation level is not sufficient to dismantle the cell, and therefore only results in more selective targeting of substrates.

Caspase-1

Caspase-1 is best known for its role in inflammation, where it cleaves pro-IL-1β and pro-IL18 into their active cytokine forms (Li et al., 1995). It is also implicated in cell death. A novel form of cell death induced by invasive bacteria, the pyroptosis, has been proposed and this form of cell death is dependent on caspase-1 (Fink and Cookson, 2005).

Caspase-1 activation, the inflammasome and the pyroptosome

Structurally, caspase-1 has a long prodomain and is predicted to act as an initiator caspase. Like other initiator caspases, its activation is triggered by the assembly of a multimeric protein complex. This complex is known as the inflammasome, which resembles structurally the apoptosome. In this complex, NLR (NACHT-LRR) family members such as NALP1, 3, and IPAF serve as scaffolding proteins (Martinon and Tschopp, 2005). The activation of caspase–1 in the inflammasome is comparable to that of caspase-9 in the apoptosome (Figure 3). In general, NLR members are typically composed of three domains: a Leucine Rich Repeat (LRR) ligand sensing domain, a NACHT nucleotide binding and oligomerization domain, and a CARD or Pyrin domain (PYD) for caspase recruitment (Martinon and Tschopp, 2004; Nadiri et al., 2006).

Four types of inflammasomes have been characterized and shown to activate caspase-1: NALP-1, NALP–3, NAIP5 and IPAF. (Martinon and Tschopp, 2004; Molofsky et al., 2006). The interaction between NALP proteins and caspase-1 is not direct but depends on the adaptor ASC; whereas, IPAF can activate caspase-1 directly via its CARD

(Mariathasan et al., 2006). Recently some natural stimuli of the inflammasome have been identified. The NALP-3 inflammasome stimuli include bacterial RNA ((Kanneganti et al., 2006), uric acid crystals associated with Gout (Martinon et al., 2006), extracellular ATP, the toxin Nigericin and the calcium channel affecting marine toxin maitotoxin (Zamboni et al., 2006); the NAIP5 inflammasome is stimulated by the bacteria *Legionella pneumophila* (Fortier et al., 2006; Zamboni et al., 2006); The IPAF inflammasome is activated by *Salmonella* (Mariathasan et al., 2004) and by cytoplasmic flagellin (Miao et al., 2006).

More recently, elegant work by Alnemri and colleagues have introduced a second caspase-1 activating complex, which they termed the "pyroptosome". The pyroptosome is solely composed of ASC dimers and is devoid of any NLR. It is hypothesized when the activation of caspase-1 in the inflammasome is subtle, it results in controlled inflammation; while activation of caspase-1 by pyroptosome is more complete, which leads to cell death or pyroptosis. We hypothesize that pyroptosis is executed by the "hyperactive" caspase-1 through cleaving a wider range of cellular substrates.

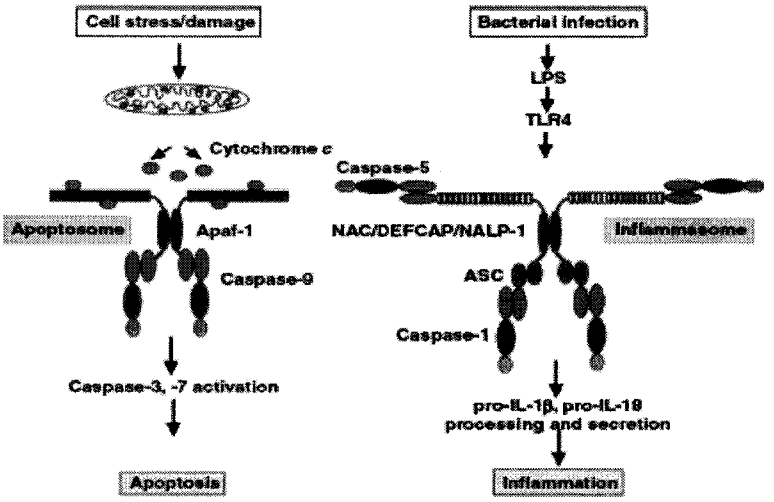


Figure 3 Apoptosome vs. Inflammasome (Creagh, Conroy et al. 2003)

Caspase-1 function

Caspase-1 was originally known as the interleukin-1β-converting enzyme (ICE), as it processes pro-IL-1β to its active form. Now we know that it also acts on IL-18 and IL-33.

The importance of caspase-1 in inflammation is highlighted by the fact that the cytokines that it activates have been implicated in the pathophysiology of various diseases, including rheumatoid arthritis, septic shock and inflammatory bowel disease (Dinarello and Wolff, 1993). The role of caspase-1 in inflammation is fully supported by the *in vivo* study that caspase-1 knockout mice are defective in the production of mature IL-1β, IL-1α and IL-18 as well as the downstream cytokines IL-6 and interferon-γ, and are highly resistant to endotoxic shock (Kuida et al., 1995; Li et al., 1995). On the contrary, these mice as expected are susceptible to bacterial infections with *E. coli* and *Salmonella* (Joshi et al., 2002; Lara-Tejero et al., 2006).

Caspase-1 is not involved in apoptosis due to the fact that caspase-1-deficient mice have no apparent defects in developmental programmed cell death and caspase-1 deficient cells respond normally to most apoptotic stimuli (Earnshaw et al., 1999). However, caspase-1 activation in macrophages infected with *Salmonella* or *Shigella* causes massive cell death in host (Fink and Cookson, 2006). This form of cell death is termed as pyroptosis as it is related to inflammation and is distinct from apoptosis, which actively inhibits inflammation.

Except for caspase-1 dependent cell death in the immune system, caspase-1 function is also implicated in cell death in central neuron system and cardiac circulation systems. Mice deficent in caspase-1 are resistant to neonatal hypoxic-ischemic brain damage (Liu et al., 1999). Transgenic mice that express a catalytically inactive caspase-1 (C285G) mutant inhibit trophic factor withdrawal-induced apoptosis in dorsal root cells; similar results were obtained in neurons isolated from newborn caspase-1 knockout mice (Friedlander et al., 1997b). As well, transgenic mice with overexpresion of caspase-1 in heart have shown strikingly increased cardiac myocyte cell death under ischemia/reperfusion injury (Syed et al., 2005). This study demonstrates that

cardiomyocytes can respond to caspase-1 overexpression and its subsequent activation to initiate cell death under certain stresses.

In contrast to pyroptosis caused by activation of caspase-1 in macrophages, a novel function of caspase-1 has been proposed in cell survival (Saleh, 2006). In response to a drop in cytosolic [k^+], caspase-1 is activated via IPAF and ASC/NALP3 inflammasomes, followed by activation of central regulators of membrane biogenesis, the Sterol Regulatory Element Binding Proteins (SREBPs), which in turn promote cell survival (Gurcel et al., 2006). Interestingly, other caspases are also implicated in this pathway: SREBP1/2 were shown to be cleaved and activated by caspases (Wang et al., 1995; Wang et al., 1996) and Caspase-2 was found to be a transcriptional target of SREBP2 (Logette et al., 2005). These findings link lipid metabolism to cell death and reveal a mechanism by which cells regulate the balance between survival and death pathways in response to pathogen invasion.

Negative regulation of caspase-1 by caspase-12

Caspase-12 has been shown to negatively regulate caspase-1 both in humans and rodents (Saleh et al., 2004). A polymorphism in caspase-12 results in the production of either a truncated variant (caspase-12S) or a full-length variant (caspase-12L). Most individuals express caspase-12S, and only about 20% of individuals of African descent express caspase-12L. Those individuals expressing caspase-12L have hypo-responsiveness to LPS-mediated production of cytokines such as IL-1β and INFγ and have an increased risk of developing severe sepsis (Saleh et al., 2004). Like the human full-length variant of caspase-12, murine caspase-12 also abrogates the inflammatory response (Saleh et al., 2006). Caspase-12 deficient mice are more resistant to sepsis and are able to clear bacterial pathogens more efficiently than wild-type mice. This may largely be due to the inhibitory effect caspase-12 has on caspase-1 (Scott and Saleh, 2007).

Caspase-1 substrates

Caspase-1 is an inflammatory caspase that is responsible for maturation of pro-inflammatory cytokines. Although, *in vitro*, caspase-1 was shown to process the kinase PITSLRE (Beyaert et al., 1997), actin (Chen et al., 1996), parkin (Kahns et al., 2003) and PARP (Gu et al., 1995), cleavage of these substrates occurs by other caspases *in vivo*. Pro-IL-1β and pro-IL-18 are the best-known caspase-1 substrates. They are synthesized as biologically inactive forms, which lack a leader peptide. It has been suggested that the precursors of IL-1β and IL-18 enter the specialized secretory lysosome together with components of the inflammasome, following caspase-1 activation. The secretory lysosome fuses with the cell membrane and the caspase-1-processed cytokines are released as active cytokines (Pizzirani et al., 2006). IL-33 has recently been identified as a caspase-1 substrate. Mature IL-33 binds to ST2 (an orphan IL-1 receptor) and results in the activation of NF-κB and MAPK that drive production of type 2 cytokines (e.g. IL-4, IL-5 and IL-13) from polarized Th2 cells. The induction of these type 2 cytokines by IL-33 *in vivo* is attributed to severe pathological changes observed in mucosal organs (Schmitz et al., 2005).

We now known that caspase-1 has roles in cell death as well as in cell survival in addition to its well-established role in the maturation of pro-inflammatory cytokines. To fulfill these additional roles, caspase-1 must cleave proteins other than the precursors of IL-1β, IL-18 and IL-33. The identification of caspase-1 substrates is essential for our understanding of its wide range of actions.

GOAL OF THIS STUDY

Cleavage of caspase substrates is central to the function of caspases. To date, more than 280 caspase substrates have been identified. (Fischer et al., 2003). The consequence of substrate cleavage greatly advanced our understanding of the role of caspases in apoptosis. As we know, caspase-3 has a predominant role in apoptosis, but growing evidence indicates a non-apoptotic role of caspase-3 as well (Launay et al., 2005). The identification of novel caspase substrates would provide us with insights into caspase functions both in apoptosis and non-apoptotic processes.

Unlike caspase-3, only few caspase-1 substrates have been identified, despite the fact that it was the first caspase to be cloned. These substrates include the pro-inflammatory cytokines pro-IL-1β and pro-IL-18, giving caspase-1 its designation as an "inflammatory caspase". Caspase-1, however, has been shown to induce cell death, namely macrophage pyroptosis in response to bacterial infection (Monack et al., 2001). Recently, the mechanism by which caspase-1 is activated has been characterized but the precise mechanism(s) by which caspase-1 initiates/executes cell death is still unclear. Identification of caspase-1 substrates is thus critical for completing our knowledge in this respect.

The diagonal gel approach is a proteomic technique for screening for protease substrates. In this study, we applied this approach to identify substrates of caspases-1 and -3.

MATERIALS AND METHODS

Cell culture

THP-1 cells were cultured in RPMI 1640 (Invitrogen) supplemented with L-glutamine, 10 % heat inactivated fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (100μg/ml). Cells were grown in a humidified incubator at 37° C, with 5% CO_2 and maintained at density 1 X 10^6 to 1.5 X 10^6. Confluent Cells were split by diluting 1: 3 with fresh culture media.

HEK293T and HeLa cells were cultured in DMEM (Invitrogen) supplemented with L-glutamine, 10 % heat inactivated fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100μg/ml). Cells were grown in a humidified incubator at 37° C with 5% CO_2. Confluent cells were split by trypsinization and by seeding 2.5 x 10^6 to a new 10 cm cell culture dish

Human PBMC isolation and proliferation

Human PBMCs were isolated from human blood cells by removing red blood cells using RBC lysis buffer (sigma). 2 x 10^7 cells were cultured in 15 ml RPMI. For proliferation, the cells were treated with 1μg/ml anti-CD3 antibody and 20 U/ml human recombinant IL-2 and cultured for 4 days in a humidified incubator at 37° C with 5% CO_2.

Diagonal gel

THP-1 cells or human PBMCs were lysed in SDS Laemmli loading buffer (50 mM Tris [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 2.5% β-mercaptoethanol) and sonicated. 400 μg of protein was resolved by SDS-PAGE 10% (first dimension). After migration, the lane containing the protein was excised and soaked in 40% EtOH and 10%

Acetic acid for 10 min, in EtOH 30% for 10 min, and then in ultrapure water for 2 x 10 min. The lane was then air dried until the gel started to curl and then soaked in CHEGG buffer (50 mM Hepes-KOH [PH 7.2], 10% glycerol, 0.1% chaps, 2 mM EDTA and 5 mM DTT freshly added) with or without 50 μg of recombinant caspase-1 or caspase-3 (Merck), and incubated overnight at 37°C. The lane was washed in water to remove the excess protease and then incubated in SDS Laemmli loading buffer for 10 min at 95°C. After cooling, the lane was then loaded on a second 10% SDS PAGE gel and resolved again. After migration, the gel was stained using Sypro Ruby (Invitrogen) or silver stain. Cleaved proteins, which were located under the diagonal, were excised from the gel and identified by mass spectrometry (at the McGill University and Genome Quebec Innovation Centre using LC-MS).

In vitro cleavage assay with whole cell lysates

Each 8 x 10^6 THP-1 cells were lysed in 100 μl CHEGG buffer and sonicated. Protein concentration was measured using Bio-Rad protein assay reagent. Each 40 μg proteins were incubated with 340 ng recombinant caspase-1 (Merck) or caspase-3 (Sigma) at 37 °C for 3 hrs, then resolved on 4-12% SDS-PAGE for western blots.

Western blot

After SDS-PAGE, the proteins on the gels were transferred to Hybond-C Extra, nitrocellulose membrane (Amersham) at 50 V for 1 hr. The blots were first incubated in blocking buffer (Tris-buffered saline, pH 7.4 (TBS), 5% (w/v) non fat milk, 0.1 % (v/v) Tween 20) for 1 hr at room temperature and then incubated for 2 hrs in primary antibody diluted in the same buffer. After washing three times each for 10 min in 1 xTBS with 0.1% (v/v) Tween 20 for 10 min, blots were incubated for 1 h at room temperature with secondary antibodies that were diluted in blocking buffer. Blots were washed three times in 1 x TBS, 0.2 % (v/v) Tween 20 for 5 min, and three times in 1 x TBS, 0.1% (v/v)

Tween 20 for 5 min. Detection was performed by chemiluminescence using ECL (Amersham).

The following antibodies were used: anti- α-enolase, aldolase, triosephosphate isomerase and caspase-1 P10 (Santa Cruz); anti- GAPDH (Abcam); anti- α-tubulin (sigma), anti-IL-1β (cell signaling).

RNA extraction and RT-PCR

Total RNA was isolated from THP-1 cells using the Trizol Reagent (Invitrogen). Each 1 x 10^6 cells were lysed in 200 μl Trizol, and extracted with 40 μl chloroform. Following centrifugation, RNA remained in the upper aqueous phase. The upper layer was removed to a fresh tube and isopropanol was added to precipitate the RNA. The RNA was dissolved in 50 μl DEPC water, and its concentration was measured by spectrometry. For first strand cDNA synthesis, 200 ng of total RNA was incubated with 1 ?g oligo $(dT)_{12-18}$ primer (500 ?g/ml, Invitrogen), 1 μl 10mM dNTP mix in a final volume of 12 μl. The reaction was heated to 65 °C for 5 min and then quickly chilled on ice to allow annealing of the oligo (dT). The mixture was further incubated at 42 °C for 2 min in 1X First-Strand Buffer (supplied with the kit), 0.01 M DTT and 40 U Rnase inhibitor (Promega). M-MLV RT (Invitrogen) was added to the mixture and incubated for 50 min at 37 °C, followed by enzyme inactivation at 70°C for 15 min.

Constructs and site-directed mutagenesis

PCR amplification of glycolysis enzymes from cDNA was carried out by mixing Taq DNA polymerase (Roche) 2.5 units, 1.5 mM $MgCl_2$ 0.8 mM dNTP, 5 μl template cDNA and 0.8 μM forward and reverse primers specific for glycolysis enzymes (table 2) in a final volume of 50 μl. PCR cycling was 2 min at 94 °C for initial denaturing, followed by 10 cycles of 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s and 20 cycles of 94 °C for

30 s, 60 °C for 60 s, and 72 °C for 60 s with a elongation of 20 s for each cycle and then elongation at 72 °C for 7 min. The PCRs were cloned to pcDNA3.1+ (neo) vector. The constructs from each clone were confirmed by sequencing.

Potential cleavage site mutations were introduced by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The primers for mutagenesis were summarized in table 3. All mutations were confirmed by sequencing.

In vitro cleavage of ^{35}S methionine-labeled substrates

^{35}S Methionine-labeled substrates were obtained by coupled *in vitro* transcription and translation using the Promega TNT reticulocyte lysate system. 0.7 μg of the cDNA constructs were incubated with T7 polymerase, rabbit reticulocyte lysates, amino acid mixture minus methionine, and ^{35}S methionine for 1.5 h at 30 °C. Cleavage of the *in vitro* transcribed and translated ^{35}S labeled products were performed by incubation at 37 °C for 4 hrs in the presence or absence of purified human recombinant caspase-1 (Merck) in CHEGG buffer. The cleavage reaction was terminated by the addition of SDS Laemmli loading buffer and resolved by SDS-PAGE, and viewed by autoradiography.

Caspase-1 activation with ATP/nigericin treatment

THP-1 cells were pre-treated with 20 ng PMA for 24 hrs, and then were treated with 100 ug LPS for overnight, followed by 20 min incubation with 5mM ATP or 20 μM nigericin and then the culture media were replaced with fresh media. The cells were cultured for an additional 3 hrs for IL-1β secretion. Cells were lysed in Laemmli loading buffer and resolved in 4-12 % SDS PAGE for Western blot.

THP-1 cells Salmonella infection

Bacterial preparation for infection

Wild-type bacteria Salmonella (SL1344) were cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar without antibiotics. Bacteria were freshly plated on LB agar. To obtain stationary-phase bacteria for infection of THP-1 cells, LB broth was inoculated with a single colony and grown overnight with vigorous shaking. Before infection, bacteria were diluted 1:10 in LB broth, grew until OD_{600} 0.9 (equivalent to 10^5 bacteria/ml), and then bacteria were harvested by centrifugation, washed with PBS, and incubated for 20 min at 37°C in THP-1 culture media (without antibiotics). The bacteria were used immediately for infection of THP-1 cells.

Cell culture and infection.

THP-1 cells were maintained at a density of 1 x 10^5 to 2 x 10^6 cells/ml. One day prior to infection, the cells were harvested, washed, and resuspended in fresh medium without antibiotics. The cells were incubated with PMA (20 ng/ml) for one overnight to differentiate the cells into adherent macrophage-like cells, The next day, medium and nonadherent cells were removed and replaced with fresh complete medium without antibiotics. The cells were then primed with 50 ng/ml crude LPS for one overnight. The next day, the cells were infected with prepared bacteria *Salmonella* at a multiplicity of infection (moi) of 10:1 bacteria: THP-1 cells. Culture plates were centrifuged at 500 x g for 10 min and incubated at 37°C for 30 min to allow phagocytosis to occur. Under these conditions, essentially all cells are infected with bacteria. The medium was then replaced with fresh medium without antibiotics and incubated for additional 16 hrs. Two parallel wells were prepared for $FLICA^{casp\text{-}1}$ analysis and western blot for each condition. The cells for western blot were lysed in SDS Laemmli loading buffer and resolved on 4-12 % SDS PAGE.

FLICA^{casp-1} activity measurement

FLICA^{casp-1} (immunochemistry Technologies) was added to cell culture at the indicated time points, followed by 1 hr incubation at 37 °C. Cells were collected and washed two times with wash buffer (supplied with the kit) and then re-suspended in the same buffer. Fluorescence was measured by FACS (BD FACS Calibur) under the FL1 channel. Results were analyzed using the software FlowJo.

LDH measurement of THP-1 cells infected with Salmonella

THP-1 cells were aliquoted into 10 cm plates at a density of 2 X 10^6 cells per plate. The cells were treated with PMA (20 ng/ml) for one overnight. After PMA treatment, the media were replaced with fresh media. The cells were infected with *Salmonella* (moi 1:100) following the *Salmonella* infection procedure. After 4 hrs of infection, the culture media were collected for LDH measurement using a kit from Trinity Biotech following its instruction. For YVAD-FMK treatment, the cells were pre-treated with YVAD-FMK (100 μM) 30 min before PMA treatment, and YVAD-FMK was kept in the culture media at all time.

Mice model of LPS-induced septic shock

C57BL/6 mice (8-12 weeks old) were injected intraperitoneally with 20mg/kg LPS from *Escherichia coli* (sterotype 055:B5, Sigma). Control mice were injected intraperitoneally with saline. The mice were killed 12 or 24 hrs post-injection. Diaphragm muscle was excised and proteins were extracted for western blot.

RESULTS AND DISCUSION

1. Caspases-1 and -3 substrates identified by the diagonal gel approach

The diagonal gel approach is a technique for protease substrate screening. It was recently applied to identify the mitochondrial caspase-3 substrate NDUFS (Ricci et al., 2004). Figure 4 schematically outlines this method. In general, whole cell lysates are separated on a 1st dimension SDS PAGE gel. The gel lane is cut, dehydrated and then rehydrated in a buffer containing the protease of interest. These steps wash out the SDS and allow the protease to diffuse through the gel pores and digest its specific substrates. Following this in-gel digestion, the gel lane is loaded horizontally on a second SDS-PAGE gel and the proteins are resolved. Most of the proteins would migrate on a diagonal line, however proteins that got digested by the protease would drop under the diagonal. To validate this method, we subjected 400 μg of proteins from whole cell lysates to the diagonal gel in the presence or absence of caspase-3. In the absence of caspase-3, all the proteins migrated along the diagonal line. In the presence of caspase-3, its substrates were cleaved and dropped below the diagonal (figure 5). Proteins were detected by staining of the diagonal gel with Syproruby (Invitrogen) or by silver staining. We further validated this technique by western blot. We loaded whole cell extracts from LPS pre-treated THP-1 cells on a 1st dimension gel and incubated the gel with caspase-1. The cleavage of the known caspase-1 substrate IL-β was monitored by western analysis (figure 6). As shown in figure 6, the 34 KDa pro-IL-**1**β was cleaved into the mature 17 KDa IL-1β by caspase-1 and dropped under the diagonal.

We chose to work with human peripheral blood mononuclear cells (PBMCs) or with the human monocytic cell-line THP-1 to screen for caspase-3, and caspase–1 substrates, respectively. 400 μg whole cell extracts were resolved on a 1st dimension SDS PAGE,

the gel lanes were incubated in the presence or absence of 50μg recombinant caspase, and then resolved again on a 2nd dimension SDS PAGE (figure 7). The proteins that dropped under the diagonal were identified by mass spectrometry (at the McGill University and Genome Quebec Innovation Centre using LC-MS). Using this approach, we identified 85 and 58 substrates for caspase-1 and –3, respectively (table 1). The criterion that we used to identify a peptide from PMF (peptide mass fingerprint) is an ion score for each peptide > 40, (which is a measure of the identity of the peptide obtained) and a peptide coverage larger than 10% of the size of the caspase-1 cleavage product which was extracted from under the diagonal. The identified substrates were classified into subgroups related to their cellular function: cytoskeletal proteins, chaperons, nuclear proteins, translation machinery proteins, immune proteins, glycolysis and bioenergetics proteins, and proteins involved in signaling and trafficking (figure 8). Among the identified substrates, 19 proteins were found to be known substrates of caspase-3 (Fischer et al., 2003), for example, cytoskeletal proteins: actin, filamin, gelsolin, keratin and vimentin; chaperons: Hsp70 and 90; proteins for RNA synthesis: hnRNP A1/A2/Cb; translation machinery proteins: EEFA1, Nascent polypeptide-associated complex, etc. (Fischer et al., 2003), which further confirmed accuracy and efficiency of this approach. Interestingly, caspase-1 and –3 shared 25 common substrates, which accounted for approximately 26% of total caspase-1 substrates and 38% of total caspase-3 substrates identified by this method (figure 9A). The overlapping substrates were mostly cytoskeletal proteins, accounting for 50% of the substrates in this subgroup (figure 9B). Among the 5 common cytoskeletal substrates, 4 are known caspase-3 substrates cleaved during apoptosis. The significant overlap of the substrates suggests that caspase-1, like caspase-3, may execute cell death. In our substrate list, immune proteins were unique for caspase-3. This may be due to the fact that we used PBMCs as a source of proteins in our caspase-3 substrate screen. This suggested that caspase-3 cleaves common proteins as well as cell-specific proteins, to fulfill its functions in apoptotic and non-apoptotic

processes. It is also worth noting that caspase-1 cleaved more proteins related to protein translation, such as translation elongation factors and ribosomal proteins, than caspase-3 did, which implies that caspase-1 could have a dominant role in destroying the translation machinery during cell death.

Although only few caspase-1 substrates are recorded in the literature, we identified a large number of caspase-1 substrates in our screen. It was not surprising since we know that the mature caspase-1 is a very active enzyme that cleaves pro-IL-1β very efficiently. Moreover, caspase-1 is very similar to caspase-3 structurally and its active site also recognizes the caspase-3 target motif, DEVD, with a binding affinity K_i = 17nM (Nicholson and Thornberry, 1997). That might explain many common substrates obtained for these two caspases. We validated eight substrates from our caspase-1 substrate list using *in vitro* cleavage assay either by western blot of whole cell lysates or by *in vitro* transcribed and translated ^{35}S labeled substrates, two of them were not cleaved, six of them were shown to be cleaved by caspase-1, which means that the chance of getting true substrates from our list is 75%.

More recently, stimuli such as bacterial and viral components as well as some toxins have been reported to activate caspase-1 via inflammasomes (Ogura et al., 2006). Our list of caspase-1 substrates provides cues for catching *in vivo* cleavage of caspase-1 substrates under those physiological conditions.

2. Caspase-1 targets in the glycolytic pathway

Glycolysis is the anaerobic catabolism of glucose, in which one molecule of glucose is catabolized into two molecules of pyruvate and two ATP molecules. Under aerobic condition, pyruvate is further oxidized by mitochondrial enzymes to CO_2 and H_2O resulting in a higher yield of ATP. In the absence of oxygen, pyruvate is converted into

lactate to regenerate NAD+ from NADH. Surprisingly, of all 11 enzymes involved in this process, 6 were found to be targeted by caspase-1 in our diagonal gel experiment (figure 10). In addition, other enzymes related to glucose metabolism were also found in our caspase-1 list including: aldose reductase, which is responsible for the conversion of glucose to sorbitol, cAMP-dependent pyruvate kinase regulatory subunit alpha 2 for glycogenolysis; fructose-1,6-bisphosphatase for gluconeogenesis; transaldolase and N-acetylglucosamine kinase of the pentose pathway.

As a major energy source, glucose metabolism is essential for cellular functions. Severe restrictions in glycolysis causes cell death, even in the presence of growth factors (Chi et al., 2000). Importantly, death as a result of glucose limitation proceeds via apoptosis (Vander Heiden et al., 2001). Cellular glucose metabolism is regulated by growth factors (Summers and Birnbaum, 1997). Cellular transformation occurs when cell survival, proliferation and metabolism become growth factor independent (Hanahan and Weinberg, 2000). Cancer cells develop strategies to shift their metabolism to glycolysis even in the presence of normal oxygen pressure (Gatenby and Gillies, 2004). This phenomenon is termed the Warburg effect (Warburg, 1930). The accelerated glycolysis confers cancer cells with a selective advantage as it ensures ATP levels compatible with demands of fast proliferation in hypoxic micro-environmental conditions. Changes in cellular metabolism are sufficient to commit a cell to death or to more proliferation. Based on studies demonstrating that glucose metabolism is implicated in cell death and survival, we have reason to speculate that these two crucial processes, glycolylsis and apoptosis, are linked.

Although caspase-1 is considered as an inflammatory caspase as its major function is the maturation of IL-1β and IL-18 (Li et al., 1995), its role in cell death was also demonstrated in certain types of cell, such as in macrophages (Fink and Cookson, 2005), fibroblasts (Miura et al., 1993), neurons (Arai et al., 2006) and mammary epithelial cells

(Boudreau et al., 1995). But the exact mechanism by which caspase-1 functions in cell death is unclear. Since many glycolysis enzymes are targeted by caspase-1, investigation of the cellular relevance of the cleavage of these substrates will help us to disclose how caspase-1 executes cell death.

2.1 validation of the cleavage of the glycolysis enzymes by caspase-1

2.1.1 Validation of the caspase-1 cleaving glycolysis in vitro by western blot

To validate the cleavage of the glycolysis enzymes by caspase-1, we performed *in vitro* cleavage assays. In these experiments, THP-1 lysates were incubated with or without recombinant caspase-1 then resolved by SDS PAGE on a 4-12% gel. The cleavage of substrates was assessed by western blot using antibodies against aldolase, α-enolase, TIM, GAPDH and IL-1β. The cleavage assay of pyruvate kinase was not done due to lack of an antibody. Cleavage of pro-IL-1β was chosen as a positive control. Aldolase migrated as a doublet with one band running slightly faster than the full-length protein (figure 11). Cleavage of α-enolase is obvious. The cleavage products are 37 KDa, 30 KDa and 20-25 KDa, suggesting more than one cleavage site may exist. Therefore the cleavage of aldolase and α-enolase was confirmed by western blot, but that of TIM and GAPDH was not detected by antibodies (figure 11).

Since caspase-1 and -3 share many common substrates, we further tested the cleavage of α-enolase and aldolase with recombinant caspase-3. We incubated THP-1 lystates with excessive amounts of recombinant caspase-3 (290 ng), but no cleavage was observed in our western blot with antibodies against α-enolase and aldolase (figure 12). The activity of the caspase-3 enzyme used (Sigma) was further tested with the selective caspase-3

fluorogenic peptide Ac-DEVD-AMC. Caspase-3 cleaved Ac-DEVD-AMC efficiently with a rate of cleavage of 3.94 x 10^6 AFU (arbitrary fluorescent units). min^{-1}. mg^{-1}. We concluded that caspase-1 cleaves α-enolase and aldolase, but caspase-3 does not.

2.1.2 Caspase-1 cleavage of in vitro transcribed and translated glycolysis enzymes

To confirm that the glycolysis Enzymes were cleaved by caspase-1 directly, we performed a second cleavage assay with ^{35}S-methionine labeled *in vitro* transcribed and translated (ITT) substrates.

To obtain the plasmids for *in vitro* transcription and translation, we first extracted mRNA from THP-1 lysates, and cDNA of THP-1 was generated by reverse transcription. α-enolase, aldolase, TIM and pyruvate kinase cDNA were cloned into pCDNA3.1, a T7 promoter containing vector. The GAPDH plasmid was purchased from **ATCC** and re-cloned into pCDNA 3.1 vector for ITT. The primers designed for PCR are listed in table 2. All plasmids used for ITT were confirmed by sequencing. Glycolysis enzyme ITT products were produced using [^{35}S] methionine and the TNT T7 coupled rabbit reticulocyte lysate kit from Promega.

^{35}S labeled ITT glycolysis enzymes were incubated with or without recombinant caspase-1 and -3 and then resolved by SDS PAGE. PARP, a substrate for both caspase-1 and -3 (Gu et al., 1995), was *in vitro* transcribed and translated and served as a positive control. ITT products of α-enolase, aldolase, GAPDH, and TIM were cleaved by caspase-1, but not by caspase-3; whileas pyruvate kinase was cleaved by both caspase-1 and -3 (figure 13). Caspase-1 strongly cleaved GAPDH, pyruvate kinase and α-enolase, but aldolase cleavage was minor in this assay. To determine the cleavage efficiency, we

digested ITT glycolysis enzymes with increased amount of recombinant caspase-1 from 10 ng to 700 ng. The cleavage of GAPDH started to be seen with 10 ng of Caspase-1, but at least 20 ng of caspase-1 was needed for the cleavage of α-enolase (figure 14). The cleavage efficiency of GAPDH and α-enolase is in the same range as that of PARP, which also needs a minimal 25 ng of caspase-1 for its cleavage *in vitro* (Gu et al., 1995). However, the amount of caspase-1 needed for pro-IL-1β processing is 0.1 ng (figure 14). So, caspase-1 cleaves pro-IL-1β much more efficiently. The PITSLRE kinase, a protein related to the master mitotic protein kinase Cdc2, is another substrate for caspase-1. Similar to pro-IL-1β, it only requires 0.25 ng of caspase-1 for its cleavage *in vitro* (Beyaert et al., 1997). Caspase-3, but not other caspases, also cleaves the PITSLRE kinase at the same site used by caspase-1. However, caspase-3 cleaves the PITSLRE kinase much less efficiently in that a minimum of 25 ng enzyme is required for the cleavage of its ITT product (Beyaert et al., 1997). *In vivo*, a study with embryonic fibroblasts from caspase-$1^{-/-}$ mice showed that the PITSLRE kinase was still cleaved to the same extent in the caspase-1 deficient fibroblasts as compared to wild type cells, in TNF-α induced apoptosis (Beyaert et al., 1997). Caspase-3 was presumably responsible for the cleavage of the PITSLRE kinase in the caspase-1 deficient fibroblasts although it cleaved this substrate less efficiently than caspase-1 *in vitro*. This demonstrates that enzymes with lower efficiency *in vitro* could also have effects *in vivo*.

An interesting phenomenon was observed with enolase: *in vitro* transcription and translation of full-length α-enolase resulted in the production of two polypeptides with molecular weights of 48 kDa and 37 kDa, respectively (figure 14 A). The 48 KDa protein is the expected size of full-length α-enolase. Further investigation disclosed that the 37 KDa protein is an alternative in-frame internal AUG translated isoform (Feo et al., 2000). The 37 KDa alternative translation product was proposed to be MBP-1, the Myc promoter-binding protein-1 that serves as a repressor of c-myc transcription (Ray and

Miller, 1991). The evidence to support this is: First, the MBP-1 cDNA shares 97% similarity with the cDNA encoding α-enolase, both in the coding region and in the 3'-untranslated sequence (Giallongo et al., 1986). Second, the chromosomal location of MBP-1 on human chromosome 1p36, is in close proximity to that of the gene encoding α-enolase (Onyango et al., 1998). Third, the 37 kDa alternatively translated isoform is preferentially localized in the nucleus, sharing with MBP-1 the capability to downregulate the transcription of c-myc (Feo et al., 2000). The antibody against α-enolase detected both the 48 KDa and 37 KDa proteins in many cell lines (Feo et al., 2000), but the antibody that we used (from Santa Cruz) only detected the 48 KDa form in THP-1 cell lysate (figure 11), whereas a 37 KDa cleavage fragment was detected in the lane digested with caspase-1 by western blot (figure 11). Comparison of α-enolase's western blot and ITT gel revealed that the cleavage product of around 20-25 KDa fragment was detected by both methods. The 37 KDa cleavage fragment, which was observed by western blot, could also exist on the ITT gel, however it would be masked by the 37 KDa alternative translation product (figure 14 A). It should be noted that caspase-1 cleaved both forms of α-enolase, the 48 KDa and 37 KDa proteins. Further work needs to be done to examine whether caspase-1 cleaves α-enolase to generate a 37 KDa fragment. This could be accomplished by blocking the internal translation of the 37 KDa isoform. Since internal translation of the 37 KDa isoform starts at Met^{97} in the α-enolase cDNA (Feo et al., 2000), point mutation of this site could possibly block internal translation. If caspase-1 cleaved α-enolase into a 37 KDa fragment, it would be interesting to examine whether this cleavage product would have similar function as MBP-1.

ITT of aldolase also showed a minor alternative translation form of around 22 KDa (figure 14 B), but this alternative translation form is not recorded in the literature. Caspase-1 cleaves both translation products, and the cleavage fragments migrate at around 28 KDa and 10 - 15 KDa.

2.2 Identification of the caspase-1 cleavage site in the glycolysis Enzymes

After we have confirmed that the glycolysis enzymes were directly cleaved by caspase-1, we then sought to map the caspase-1 cleavage sites. As caspases have a near absolute preference for Asp residues in the P1 position, we scanned the amino acid sequence in α-enolase and GAPDH for possible caspase cleavage motifs, which would generate the fragments shown in the ITT cleavage experiments (Figure 14). We used site-directed mutagenesis to change the possible Aspartate residues to Alanine in four possible positions in GAPDH and α-enolase (figure 15A, 16A). As shown in figure 15C, GAPDH mutant $D^{189}A$ was resistant to caspase-1 cleavage, whereas all four mutations in α-enolase couldn't block its cleavage by caspase-1 (figure 16 B). Inspection of the cleavage site of GAPDH from several species revealed that the cleavage site from P1 – P4 and P1' ($KTVD^{189}\downarrow G$) are completely conserved among these species (figure 15 B). We have therefore mapped D^{189} as a single caspase-1 cleavage site in GAPDH.

2.3 In vivo studies on the cleavage of the glycolysis enzymes upon activation of Caspase-1

After we have identified that those glycolysis enzymes were caspase-1 substrates *in vitro*, we then tested if these substrates were cleaved *in vivo* in response to caspase-1 activation.

2.3.1 Activation of caspase-1 by transfection of procaspase-1 into HEK293 cells

Several studies have shown that caspase-1 cleaves its substrates when over-expressed into HEK293 (Kahns et al., 2003; Lamkanfi et al., 2004; Zhang et al., 2003a). Since the glycolysis enzymes are constitutively expressed in HEK293, we transfected procaspase-1 into HEK293, but auto-processing of caspase-1 was not observed by western blot, and

α-enolase and aldolase were not cleaved (data not shown).

2.3.2 Activation of caspase-1 in THP-1 cells treated with LPS plus ATP or Nigericin

Caspase-1 is constitutively expressed in monocytes and macrophages (Lin et al., 2000). Previous research has shown that activation of caspase-1 in macrophages occurs in response to Toll-like receptor activation and ATP treatment, the latter activating the P2X7 receptor which decreases intracellular K^+ levels by forming a pore in the plasma membrane (Perregaux and Gabel, 1994; Surprenant et al., 1996). The toxin nigericin, a potassium ionophore was also found to activate caspase-1 and leads to the release of IL-1β by macrophages (Perregaux and Gabel, 1994). This process is dependent on caspase-1 activation within the NALP3 inflammasome (Mariathasan et al., 2006). We have applied this activation model to THP-1 cells. In our experiments, we pre-treated THP-1 cells with PMA to differentiate the cells into macrophages, and then treated with LPS and ATP or Nigericin. Processing of Pro-IL-1β into the 17 KDa form was shown on western blot (figure 17). Activated caspase-1 (p10) was not detected in western blot. The possible reason is that the antibody used in these experiments was raised against mouse caspase-1 and is not sensitive to human caspase-1. But the cleavage of α-enolase and aldolase was not seen by western blot. One possibility is that the activation of caspase-1 was not robust enough to cleave these substrates.

2.3.3 Activation of caspase-1 in Salmonella infected THP-1 cells

The Gram-negative bacteria *Salmonella* was reported to infect macrophages and induce cell death in the host cells by activating caspase-1 (Hersh et al., 1999). Cell death of host cells can be advantageous or detrimental to pathogenesis. In the case of *Salmonella*, the

bacteria induces cell death as a virulence strategy for its systematic spreading into the host (Monack et al., 2000). The mechanism of caspase-1 activation involves the assembly of an IPAF inflammasome (Mariathasan et al., 2006), and the consequent activation of the ASC pyroptosome (Alnemri, 2007). *Salmonella* infection-induced pyroptosis has been characterized as a caspase-1 dependent cell death as it is distinct from classical apoptosis and caspase-3 is not involved in this process (Monack et al., 2001). Therefore it was very important to investigate caspase-1 substrates cleavage during this process. We infected THP-1 cells with *Salmonella*. We first treated THP-1 cells with PMA to differentiate the cells into macrophages, primed the cells with a low dose of LPS, and then infected with *Salmonella* at different multiplicity of infection (moi). 24 hours post-infection, massive macrophage cell death was observed in culture. In this experiment, activation of caspse-1 was monitored using the fluorescent reagent FLICA^{casp1}, which binds to the active form of caspase-1 with high affinity. Our results showed that PMA treatment induced around 10% caspase-1 activation detected by FLICA^{casp1} (figure 18), however, no pro-IL-1β processing or caspase-1 auto-cleavage were observed in response to PMA. In response to LPS treatment, caspase-1 activation increased to 31%, but pro-IL-1β and caspase-1 were not yet processed. *Salmonella* infection activated caspase-1 up to nearly 100% and pro-IL-1β was processed into IL-1β. Protein level of caspase-1 is low in western blot, which is consistent with previous research showing that caspase-1 is secreted with IL-1β from the macrophage upon activation (Andrei et al., 2004). Surprisingly, α-enolase and aldolase were completely degraded (The bands detected by western blot were due to cross reactivity of the antibodies with bacterial enolase, which migrated slightly faster than mammalian α-enolase derived from THP-1). This suggests that they might be degraded in the cell after a 24 hr-infection with *Salmonella*. It has been reported that caspase substrates DIAP1 (an *Drosophila* inhibitor of apoptosis) and TWIST (a transcription factor essential for mesodermal development) are degraded by ubiquitin/proteosome pathways after

caspase cleavage (Demontis et al., 2006; Ditzel et al., 2003). In the case of DIAP1, when it is cleaved by a caspase, the N-end Asn is exposed, which sensitizes the protein to be degraded by the ubiquitin dependent N-end rule pathway (Ditzel et al., 2003). Similarly, caspase cleaves off the C-terminus of TWIST, which greatly affects stability of the protein and thus triggers its degradation by the lysine dependent ubiquitination pathway (Demontis et al., 2006). Based on the depletion of the glycolysis enzymes after *Salmonella* infection in THP-1 cells and on the results showing that the glycolysis enzymes were cleaved by caspase-1 *in vitro*, we hypothesize that the caspase-1 cleavage might target the glycolysis enzymes to ubiquitin-dependent degradation.

2.3.4 Examination of the glycolysis enzymes cleavage in the mice muscle in Septic shock

Septic shock is characterized by a systemic inflammatory response to an infection or injury, in which a group of inflammatory cytokines, such as TNFα, IL-1β, IL-6, IL-8 and IL-18 are released by inflammatory cells (Cavaillon et al., 2003; Endo et al., 2000; Grobmyer et al., 2000). Excessive activation of caspase-1 has been reported in this condition (Oberholzer et al., 2000). As we know, glucose metabolism is linked to muscle function in that ATP provides energy for muscle contraction. Therefore, Glucose metabolism, as the major source of energy in the skeletal muscle, is essential for muscle function. Patients in septic shock develop muscle weakness caused by loss of contractile ability of their skeletal muscles (Ginz et al., 2005). Posttranslational modifications of glycolysis enzymes have been observed in the diaphragm of septic rodents (Barreiro et al., 2005; Hussain et al., 2006). LPS injection in rats has been shown to cause muscle wasting: The isometric force generated by the diaphragm significantly and progressively declined in this septic shock model (Barreiro et al., 2005). We examined the cleavage of the glycolysis enzymes in the diaphragm of mice subjected to the septic shock model. For this part of the work, we collaborated with Dr. Sabah

Hussain's lab. Mice were injected intraperitoneally with LPS and killed 12 hrs or 24 hrs after injection. The diaphragms were excised to extract protein. 50 μg of total proteins were subjected SDS PAGE and probed with antibodies against α-enolase, adolase, GAPDH, TIM and actin. Actin was chosen as a loading control. Our results show that proforms of all the glycolysis enzymes examined were greatly decreased 24 hrs post LPS injection (figure 19). Activation of caspase-1 was confirmed by the detection of autocatalytic processing of caspase-1 into the p10 and p20 subunits in the 24 hrs samples (figure 19). ProIL-1β was induced 24 hrs after LPS injection, but the cleaved IL-1β was not seen due to its secretion. These results correlate with our previous results with *Salmonella* infection, in which α-enolase and aldolase were degraded 24 hrs post-infection (figure 18). The consequence of the degradation of the glycolysis enzymes would be to shut down the glycolytic energy source, leading to cell death. In future experiments, we will investigate the degradation of the glycolysis enzymes in caspase-1 knock-out mice upon LPS injection and compare the muscle contractile ability from wild type and caspase-1 knock-out mice during septic shock.

2.3.5 The Glycolytic rate is reduced during Salmonella infection of THP-1 cells

To test our hypothesis that the consequence of the cleavage of the glycolysis enzymes is to shut down this energy source and therefore promote cell death, we compared the change in the glycolytic rate of THP-1 cells with and without *Salmonella* infection. In this experiment, we pre-treated THP-1 cells with PMA and infected with salmonella for 4 hrs. The glycolytic rate of THP-1 cells was assessed by measuring lactate production in the presence or absence of the caspase-1 inhibitor YVAD-FMK. As shown in figure 20, lactate production decreased by 2 fold after THP-1 cells were infected with *Salmonella*, and caspase-1 inhibitor YVAD-FMK partially reversed this effect. Our results have

demonstrated that glycolysis is impaired in THP-1 cells infected with *Salmonella*, and that caspase-1 is involved in this process.

CONCLUSIONS AND FUTURE DIRECTIONS:

We have applied a proteomic approach, the diagnonal gel method, to screen for caspase substrates. This method is based on in-gel digestion of substrates by caspases and allows identification of caspase substrates from whole cell extracts. In our experiments, we have identified 85 and 58 potential substrates for caspases-1 and –3, respectively. We have validated 8 selected caspase-1 substrates using *in vitro* cleavage assays. Among the 58 caspase-3 substrates that we obtained, 19 substrates have been reported in the literature as bona fide caspase-3 substrates. Our results have also demonstrated that caspases-1 and –3 share 25 common substrates, which account for approximately 26% of total caspase-1 substrates and 38% of total caspase-3 substrates identified by this method. Those caspase-1 and –3 substrates are implicated in basic cellular functioning and survival (maintenance of the cytoskeleton, translation, glycolysis, bioenergetics, signaling and trafficking).

Another proteomic method, the two-dimensional gel electrophoresis (2D), has been applied to search for caspase substrates. In this method, proteins derived from healthy or apoptotic cells were separated using 2D gels and the migration patterns of proteins from a healthy versus apoptotic cell were compared (Gerner et al., 2000). Although this method is also efficient, as few hundred altered protein spots were detected on the 2D gels (Gerner et al., 2000), it has the limitation that many substrates of other proteases were also detected. However, if we combine the results from the diagonal gel and 2D gel approaches, the overlapping substrates from these two methods would represent potential true *in vivo* caspase substrates. In addition, one advantage of our *in vitro* screening is that it could uncover substrates that are undergone degradation soon after their cleavage by caspases *in vivo*. Since those substrates are quickly degraded upon apoptotic stimulation, it is impossible to catch them with *in vivo* screening methods.

Our data have indicated that the glycolysis pathway is targeted by caspase-1. Five glycolysis enzymes, α-enolase, aldolase, TIM, GAPDH and pyruvate kinase, are cleaved by caspase-1. The processing of the above glycolysis enzymes by caspase-1 has been confirmed by incubating nanogram amounts of purified recombinant human caspase-1 with ^{35}S-methionine-labelled *in vitro* transcribed and translated substrates. Additionally, we have identified the caspase-1 cleavage site in GAPDH as aspartate 189. GAPDH with a point mutation of Asp189→Ala (D^{189}-A) is resistant to cleavage by caspase-1 *in vitro*. *In vivo* cleavage as well as the consequence of this cleavage remains to be investigated.

We have examined the *in vivo* cleavage of the glycolysis enzymes in three caspase-1 activation systems: 1) Stimulation of THP-1 cells with LPS plus ATP or nigericin. This condition has been shown to activate caspase-1 within the NALP-3 inflammasome and results in the processing and secretion of the caspase-1 substrate IL-1β. In this system, we didn't detect any change in the glycolysis enzyme levels or processing. 2) *Salmonella typhimurium* infection of THP-1 cells. This condition has been reported to activate caspase-1 within an IPAF inflammasome. In both conditions we failed to observe caspase-1 activation and processing by western blot, but we detected high FLICAcasp-1 staining in response to 24 hour-*Salmonella* infection, at which point the cells were in an advanced phase of cell death and glycolysis enzymes that we examined were degraded. 3) Septic shock mice model with LPS induced endotoximia. In this condition, auto-processing of caspase-1 was detected and the protein level of the glycolysis enzymes, including α-enolase, aldolse, GAPDH and TIM were greatly decreased after 24 hr LPS injection although cleavage products of these substrates were not observed. This result correlates with that we obtained in *Salmonella* infection. In combination with our results of the cleavage of the glycolysis enzymes by caspase-1 *in vitro*, we can speculate that

those glycolysis enzymes could be degraded after cleavage by caspase-1 in the above two conditions. Indeed, caspase substrates are reported to be degraded after cleavage (Demontis et al., 2006; Ditzel et al., 2003; Du et al., 2005). Caspases initiate degradation of their substrates and target them to be recognized by the ubiquitin/proteosome system. Accumulating evidences link the ubiquitin/proteosome pathway to caspase function and many researchers have implied a function of caspase-3 in the degradation of proteins under pathogenic conditions (Mitch, 2007; Ottenheijm et al., 2006). The involvement of caspase-1 in the degradation events has not been previously reported.

In vivo cleavage of certain substrates by caspases is cell type specific and stimulus-specific. For example, GATA-1 is cleaved by caspases in erythroid cells undergoing apoptosis in response to erythropeitin (EPO) deprivation or death receptor stimulation ((De Maria et al., 1999), but remains uncleaved in erythroid cells undergoing terminal differentiation (Zermati et al., 2001); actin can be cleaved by caspases in U397 leukemia cells, neurons and thymocytes, but not in other cell types undergoing apoptosis (Earnshaw et al., 1999). So, this raises the difficulty of capturing the *in vivo* cleavage event of a known *in vitro* caspase substrate.

In addition to the above-described caspase-1 activation systems, few other caspase-1 activation systems remain to be tested and in which we could examine the cleavage of the glycolysis enzymes. Caspase-1 knockout mice develop normally and appear healthy and fertile, but thymocytes from caspase-1 deficient mice are more resistant to apoptosis induced by anti-CD95 antibody (Kuida et al., 1995; Li et al., 1995). This suggests a non-redundant function of caspase-1 in this cell type. It would be worthwhile to explore the cleavage of the glycolysis enzymes in thymocytes in response to various apoptotic stimuli.

Although the role of caspase-1 in development is minor, its role in neuronal pathological cell death appears to be significant and non-redundant. Dorsal root ganglion neurons in caspase-1 deficient mice are partially resistant to apoptosis induced by nerve growth factor deprivation (Friedlander et al., 1997b). Excessive apoptosis plays an important role in neurodegenerative diseases as well as following cerebral ischemia and head trauma (Friedlander et al., 1997a; Friedlander et al., 1997b; Hara et al., 1997). There is ample evidence that suggests that caspase-1 plays a unique role in neuronal apoptosis (Friedlander et al., 1997b). As well, it has been also proposed that caspase-1 acts as an apical mediator of apoptosis during hypoxia-induced neuronal cell death (Zhang et al., 2003a).

Hypoxia is a condition of low level of oxygen in tissues. A major intracellular adaptation to severe hypoxia is the transition from oxidative phosphorylation to glycolysis as the principal means of generating ATP. Under this condition, the hypoxia-inducible factor (HIF) pathway is switched on. In order to obtain enough ATP, cells increase their rate of glycolysis through stabilization of HIF, which induces the expression of the glucose transporter GLUT1 as well as that of certain glycolysis enzymes (Semenza et al., 1994). All of the five glycoysis enzymes that are targeted by caspase-1 are known to be HIF-1 transcriptional targets during hypoxia (Graven et al., 1999; Semenza et al., 1996). The role of glycolysis enzymes becomes essential for cell survival in this condition. It is therefore important to investigate the function of caspase-1 on these glycolysis enzymes in this context.

Tumor cells display a high rate of glucose uptake and glycolysis even under normal oxygen pressure (Warburg, 1930). Accelerated glycolysis ensures ATP levels compatible with demands of fast proliferating tumor cells in hypoxic environment. This metabolic strategy is commonly observed across tumors (Gatenby and Gillies, 2004). The increased

glucose uptake could play a role in protection from apoptosis by rendering tumor cells independent of growth factor (Vander Heiden et al., 2001).Inhibition of glucose metabolism enhances death receptor mediated apoptosis in tumor cells (Munoz-Pinedo et al., 2003). One strategy for tumor cell propagation is through repression of caspases (Kurokawa et al., 1999; Teitz et al., 2001). Since we have shown that caspase-1 cleaves glycolysis enzymes *in vitro*, we would ask a question: Do tumor cells block caspase-1 activation to maintain their glycolytic advantage? To address this hypothesis, we can try to induce activation of caspase-1 in tumor cells and examine the cleavage of glycolysis enzymes and the relevance of these cleavage events to apoptosis. Previous researchers have shown that caspase-1 dependent apoptosis is induced by doxorubicin treatment of MCF-7 cells (a breast carcinoma cell line) (Thalappilly et al., 2006). We can use this system to test our hypothesis.

Figure 4

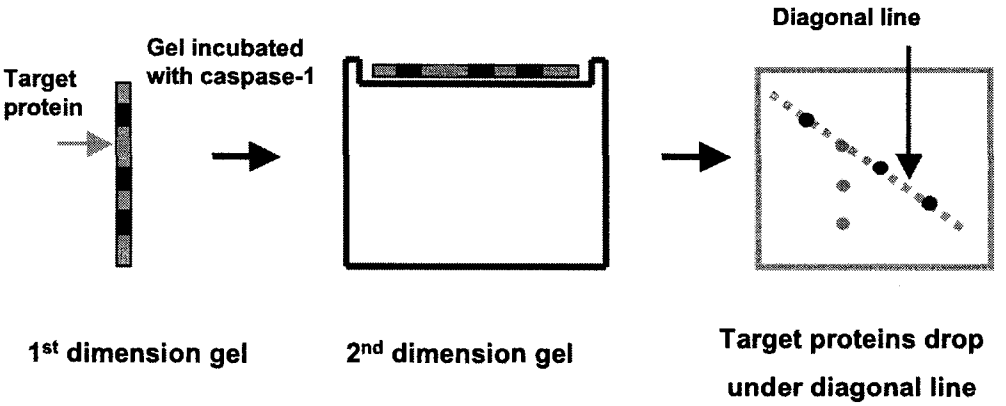
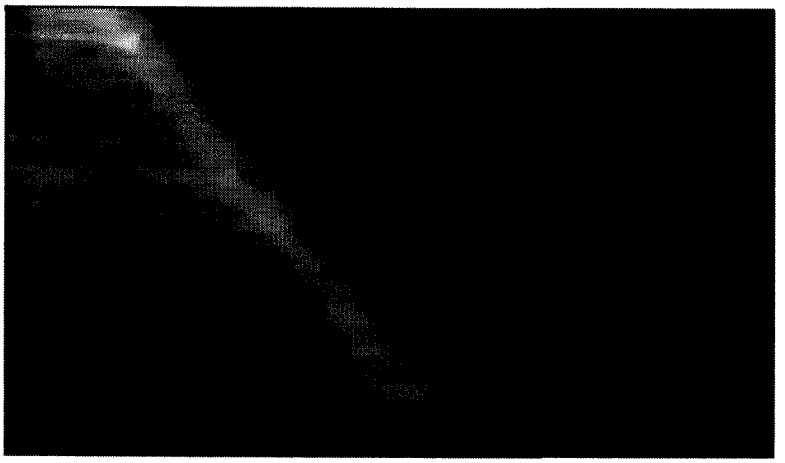


Figure 4 Schematic of Diagonal Gel Approach

Whole cell lysates are separated in 1st dimension SDS-PAGE, gel lanes are excised and dehydrated, and then re-hydrated in presence of protease of interest, allowing in-gel cleaving of substrates by protease. The processed gel lanes are further resolved by 2nd dimension SDS-PAGE. The majority of proteins migrate along diagonal line, whereas fragments of substrates cleaved by protease drop under the diagonal line. The dropped proteins are identified by MALDI-TOF.

Figure 5

A **No caspase**



B **Caspase-3**

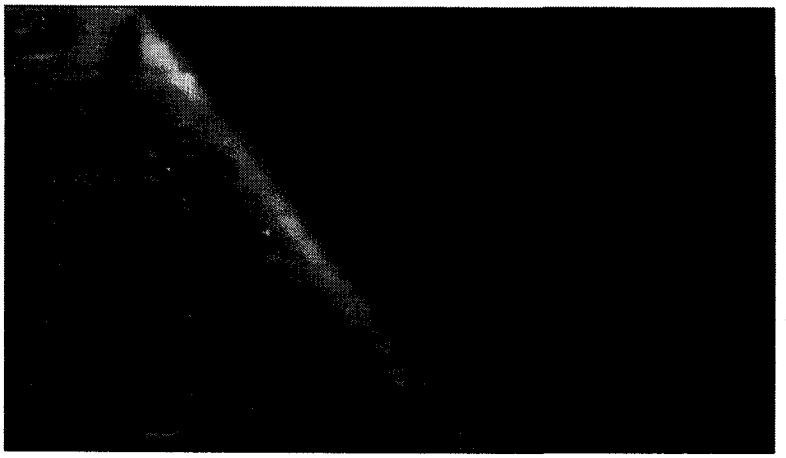


Figure 5 Diagonal Gel with Caspase-3

Whole cell extracts from PBMC were loaded on gel and ran in 1st dimension SDS-PAGE. The gel lane was incubated with or without caspase-3 for overnight, then ran in 2nd dimension SDS-PAGE. The gels were stained with Sypro Ruby and viewed under UV light. (A) without caspase (B) with caspase-3

Figure 6

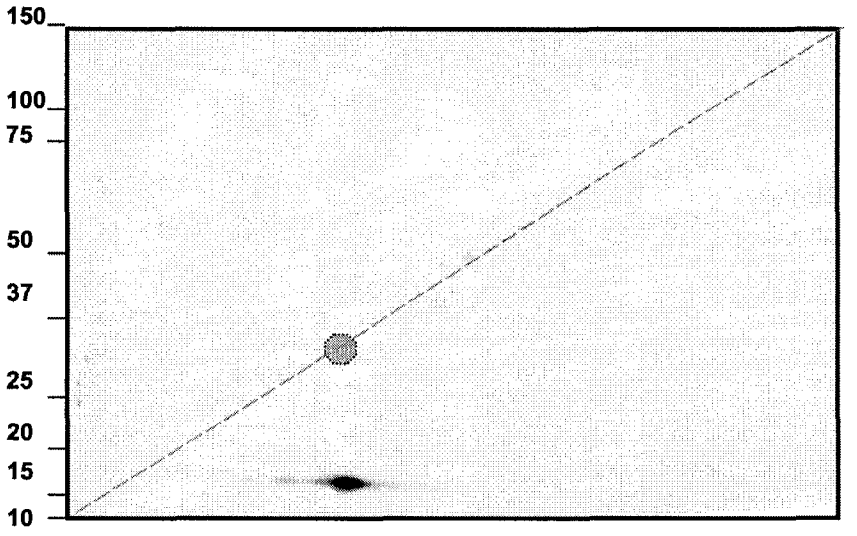


Figure 6 Caspase-1 Substrate IL-1 β Revealed by Western Blot in Diagonal Gel

THP-1 cells were pretreated with LPS for overnight, 400 μg proteins from whole cell lysates were resolved in 4-12% SDS-PAGE, the lane was hydrated and then incubated with human recombinant Caspase-1 (25μg) overnight, and again resolved with 4-12% SDS-PAGE gel, western blot using IL-1 β antibody. The dash line represents diagonal. The dot drew on diagonal line represents the proform of IL-1 β (34 KDa).

Figure 7

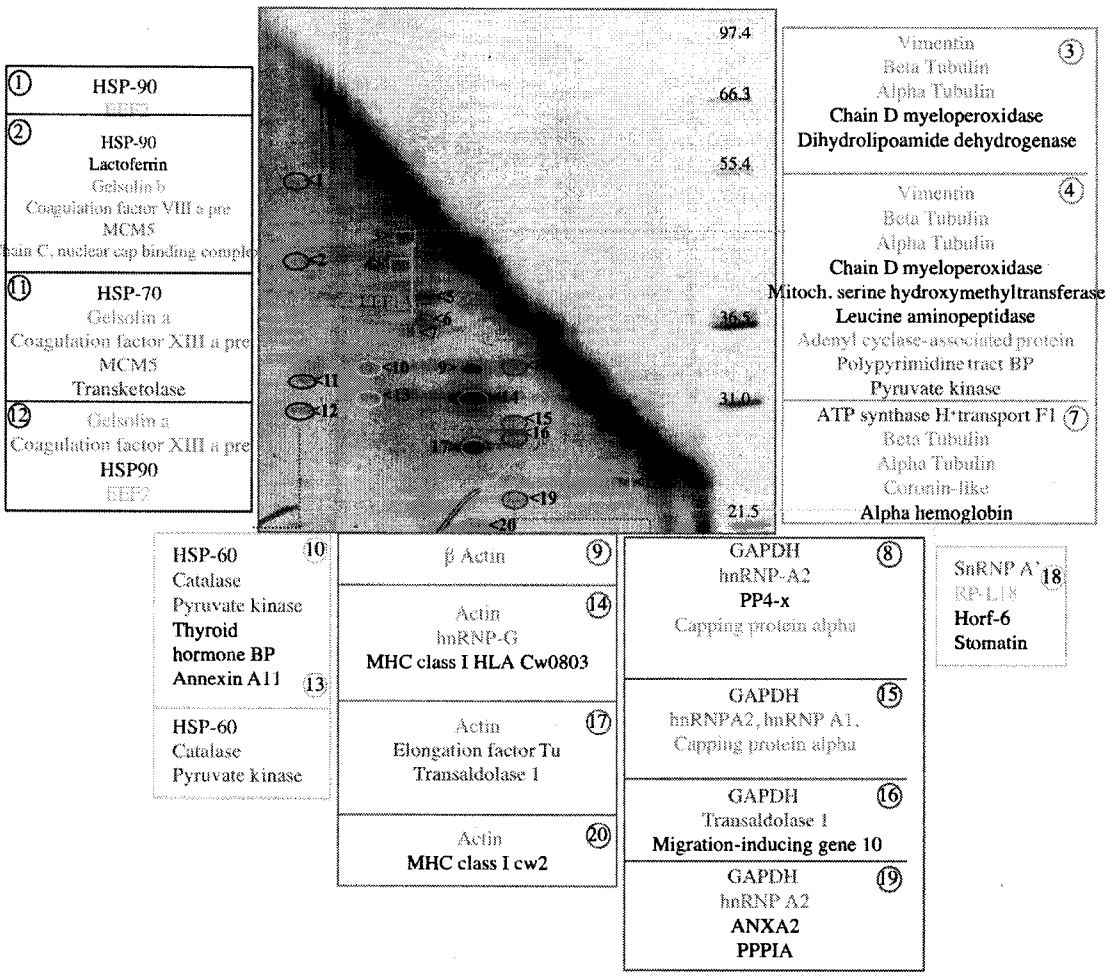
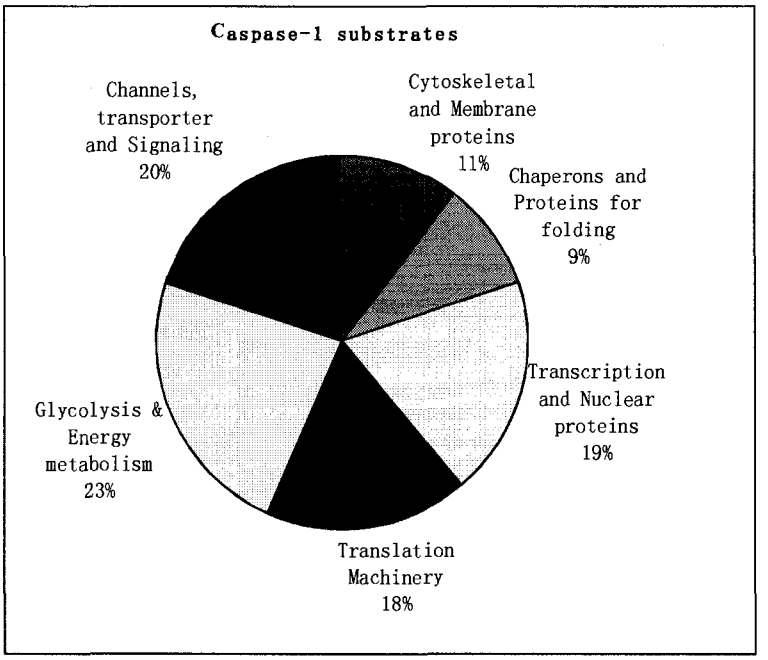


Figure 7 Map of Caspase-3 Digested Diagonal Gel

Whole cell extracts from PBMC were subjected to SDS PAGE gel and then digested with recombinant caspase-3. 2nd dimension gel was stained with silver. The dots marked were analyzed by MALDI-TOF.

Figure 8

A



B

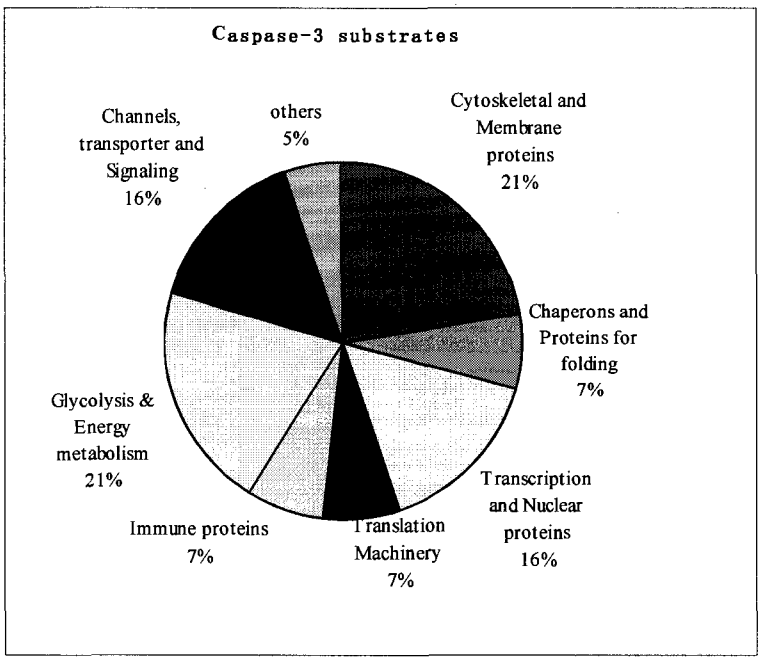


Figure 8 Pie Chart of Caspase-1, -3 Substrates

(A) Caspase-1 substrates (B) Caspase-3 substrates

Figure 9

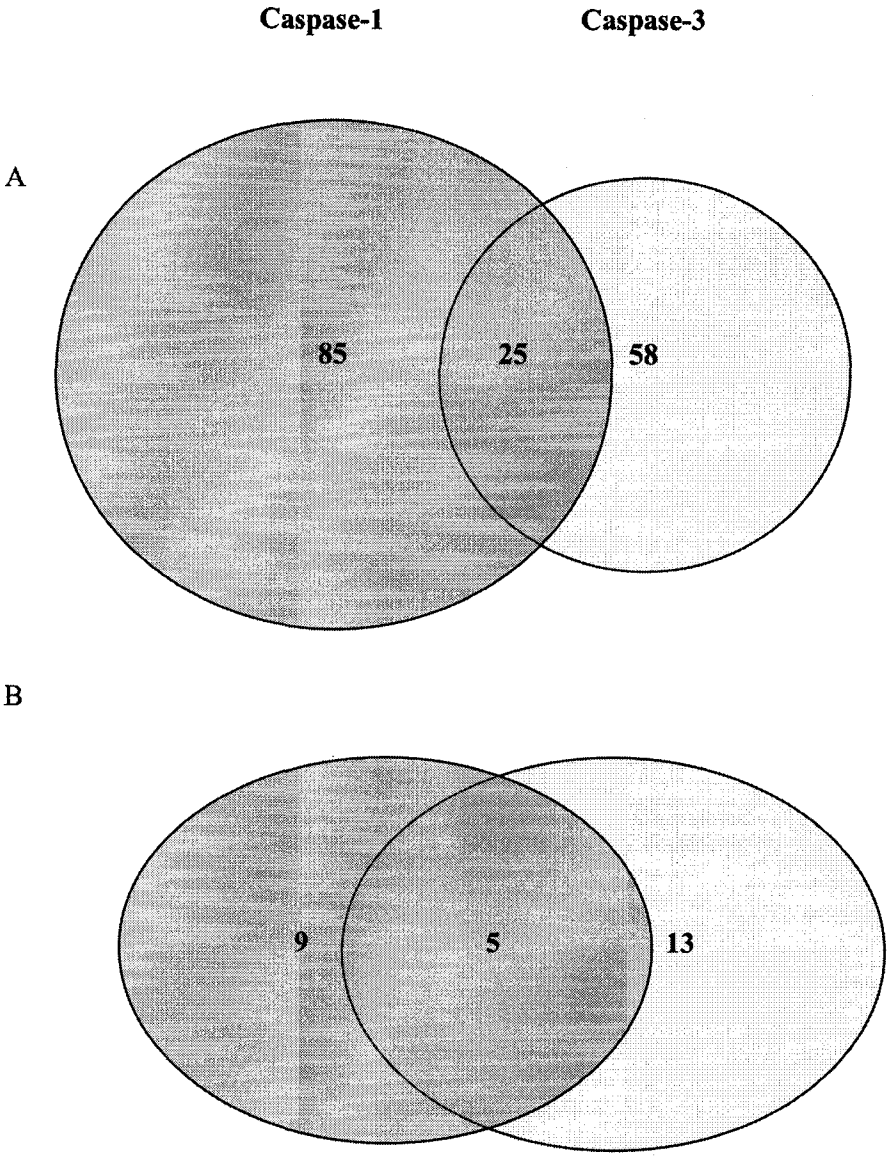


Figure 9 Venn Diagram Comparing Caspase-1 and Caspase-3 Substrates

(A) Overlapping of caspase-1, caspase-3 substrate in total proteins identified by diagonal gel approach

(B) Overlapping of caspase-1, caspase-3 substrates in cytoskeletal proteins identified by diagonal gel approach

Figure 10

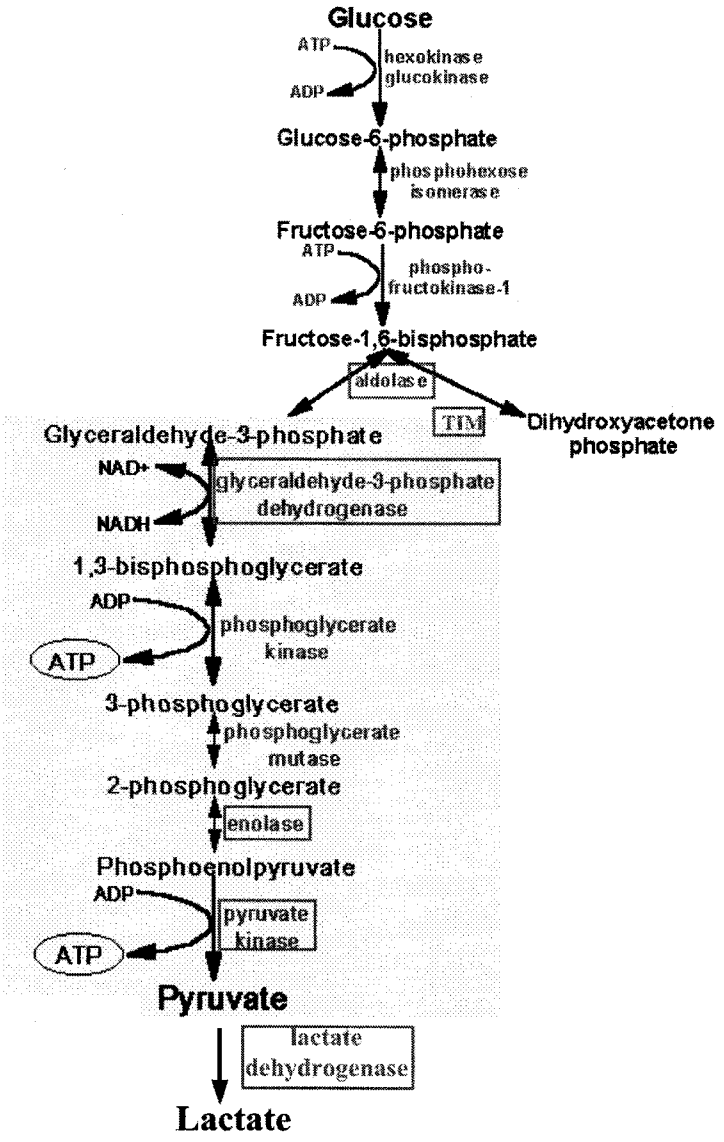


Figure 10 Caspase-1 Targeted Substrates in Glycolytic Pathway

Glycolytic pathway converts glucose to pyruvate via glycolysis enzymes. Under anaerobic condition, pyruvate is converted to lactate to re-generate NAD^+. In this pathway, 6 out of total 11 glycolytic enzymes are among our list of casapase-1 substrates, which are marked with red square.

Figure 11

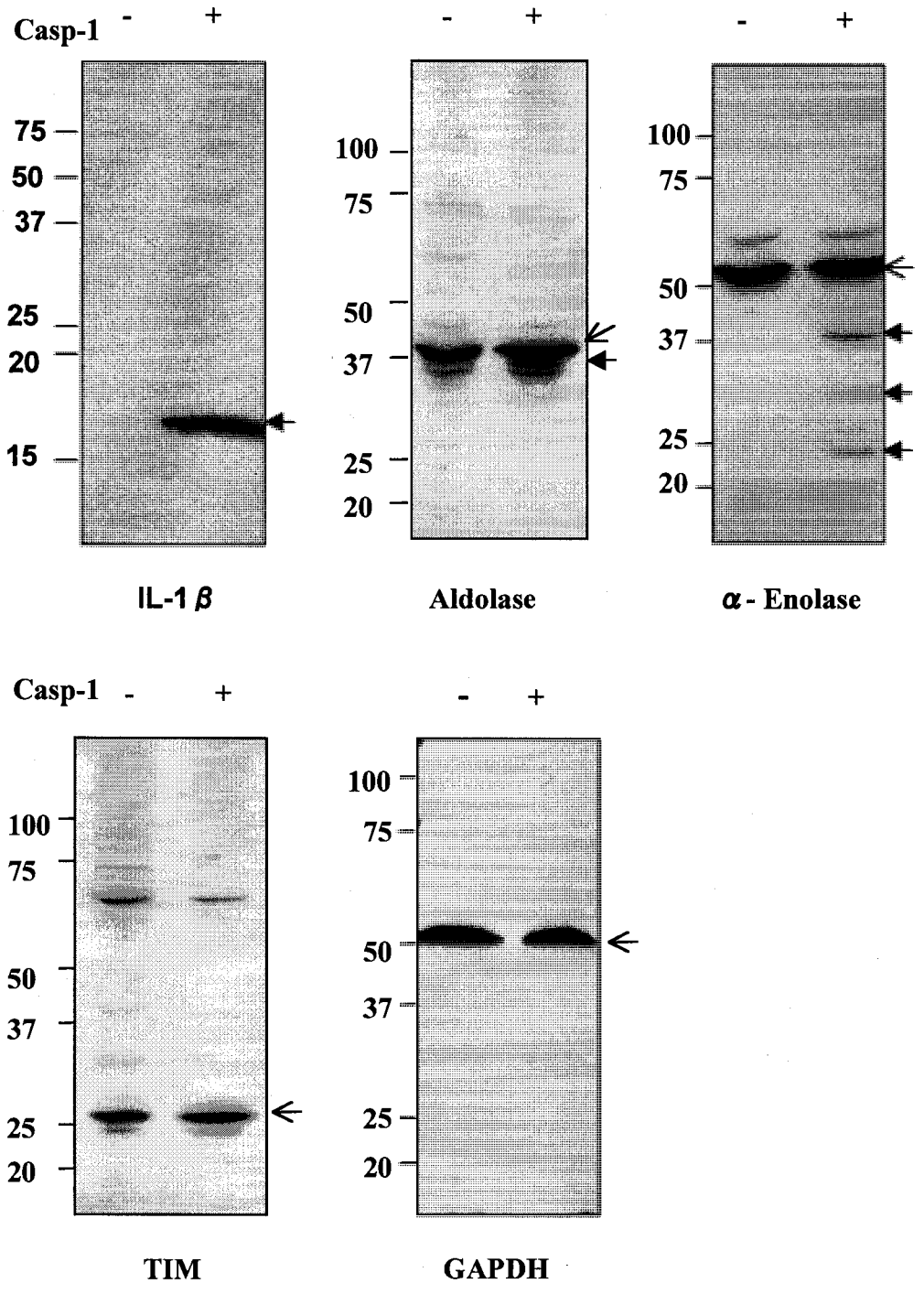

Casp-1
-
+
75
50
37
25
20
15
IL-1 β
100
75
50
37
25
20
Aldolase
α - Enolase
TIM
GAPDH

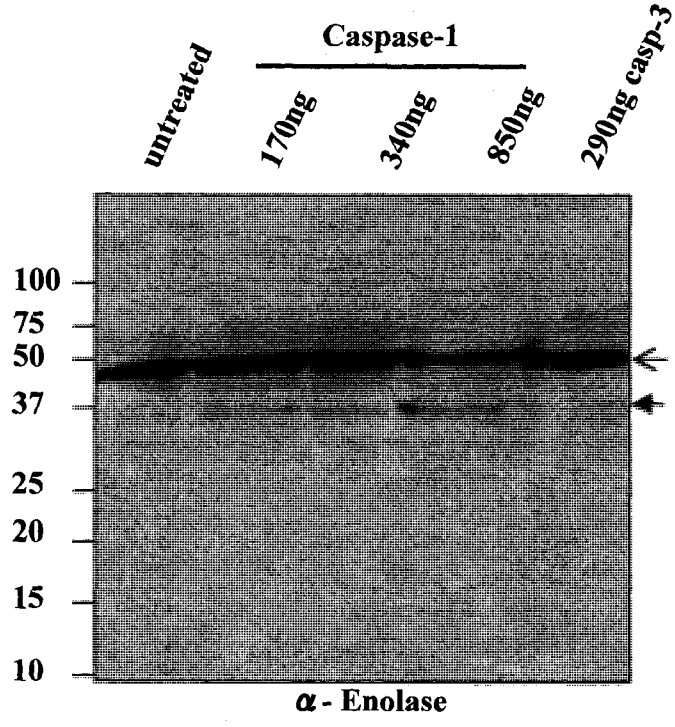
Figure 11 Western Blot of *in vitro* Cleavage of Glycolysis Enzymes by caspase-1

Whole cell extracts from THP-1 were incubated with or without recombinant caspase-1 for 3 hours at 37℃ and resolved in 4 – 12% SDS-PAGE, western blot uses antibodies against mature IL-1 β , aldolase, α - enolase, TIM and GAPDH. Open arrows indicate full-length proteins, and closed arrows indicate the cleavage fragments.

F

Figure 12

A


Caspase-1
untreated
170ng
340ng
850ng
290ng casp-3
100
75
50
37
25
20
15
10
α - Enolase

B

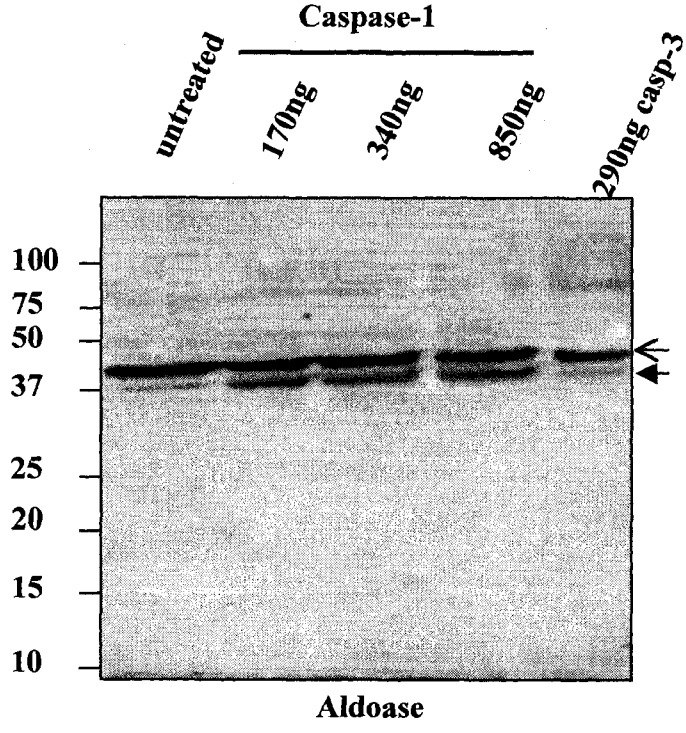

Caspase-1
untreated
170ng
340ng
850ng
290ng casp-3
100
75
50
37
25
20
15
10
Aldoase

Figure 12 Western blot of Comparing Cleavage of Glycolysis Enzymes by Caspase-1 and –3

Whole cell extracts from THP-1 cells were incubated with or without caspase-1/ -3 of indicated amount for 3 hours at 37 ℃, and resolved in 10% SDS-PAGE, cleavgage of α - Enolase and aldolase was assessed by western blot. Open arrows indicate full-length proteins, and closed arrows indicate the cleavage fragments.

(A) anti- α -Enolase

(B) anti- aldolase

Figure 13

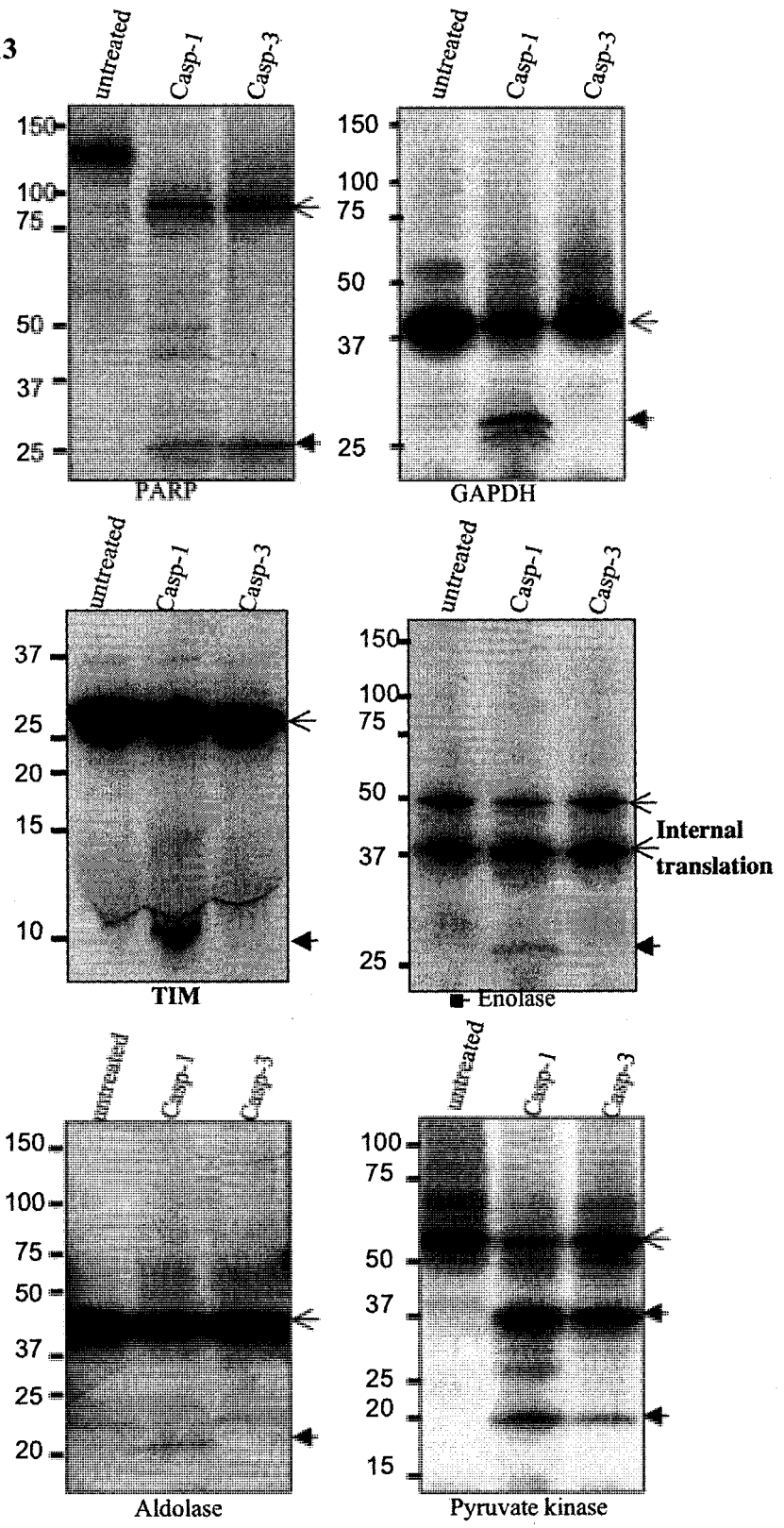

untreated
Casp-1
Casp-3
150
100
75
50
37
25
PARP
GAPDH
TIM
Enolase
Internal translation
Aldolase
Pyruvate kinase

Figure 13 Comparison of Cleavage of ITT Glycolysis Enzymes by Caspase-1 and -3

in vitro transribed and translated ^{35}S labeled PRAP, GAPDH, TIM, α - Enolase, aldolase and pyruvate kinase were incubated with or without recombinant caspase-1 (170ng) or caspase-3 (290ng) for 4 hours at 37 ℃, resolved in 4 – 12% SDS PAGE (TIM was resolved in 12% SDS PAGE) and viewed by autoradiography. Opened arrows indicate full-length proteins, closed arrows indicate cleaved fragments.

Figure 14

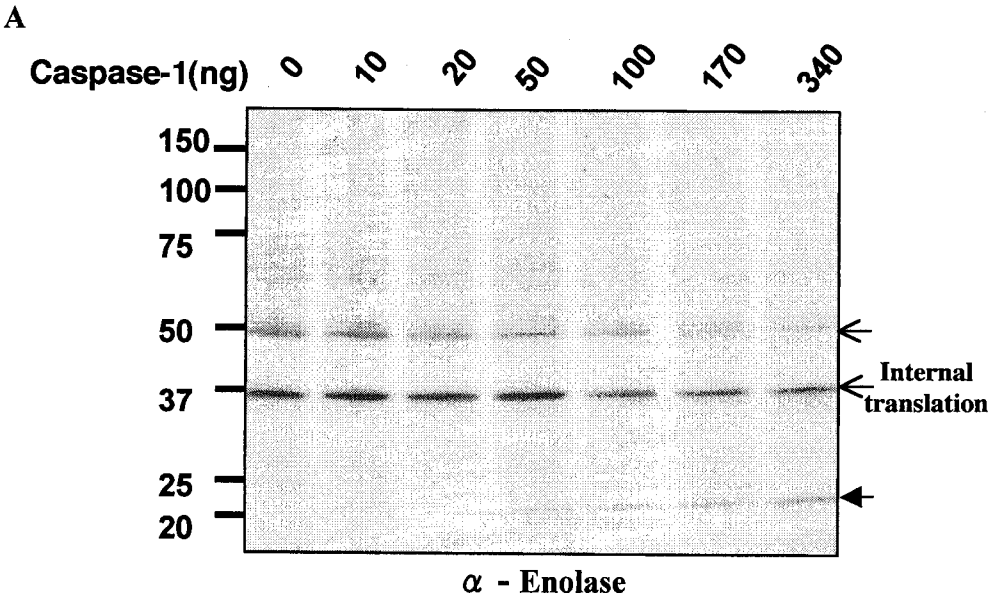
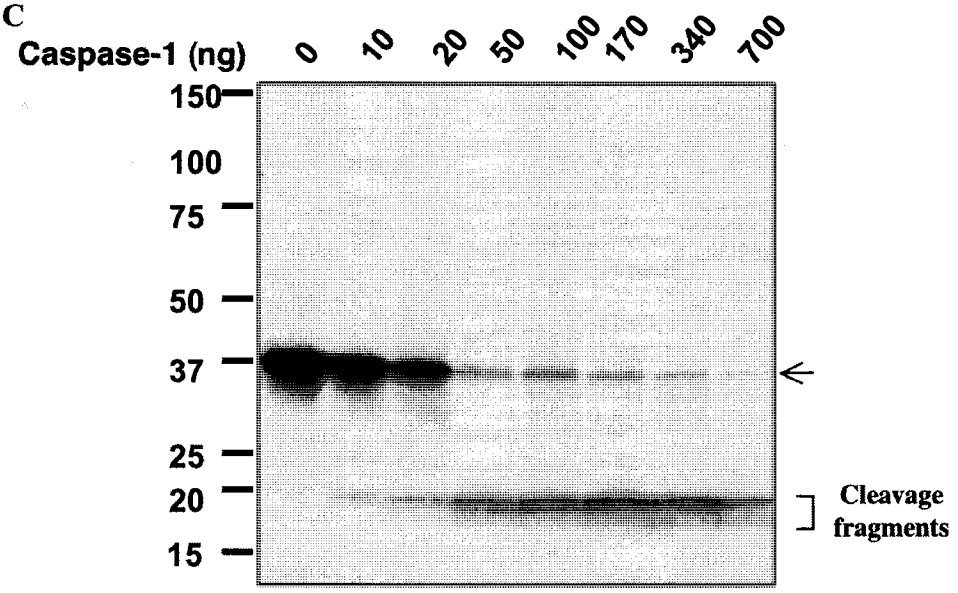

A
Caspase-1(ng)
0
10
20
50
100
170
340
150
100
75
50
37
25
20
Internal translation
α - Enolase
B
Caspase-1(ng)
0
10
20
50
100
170
340
150
100
75
50
37
25
20
Possible internal translation
Aldolase

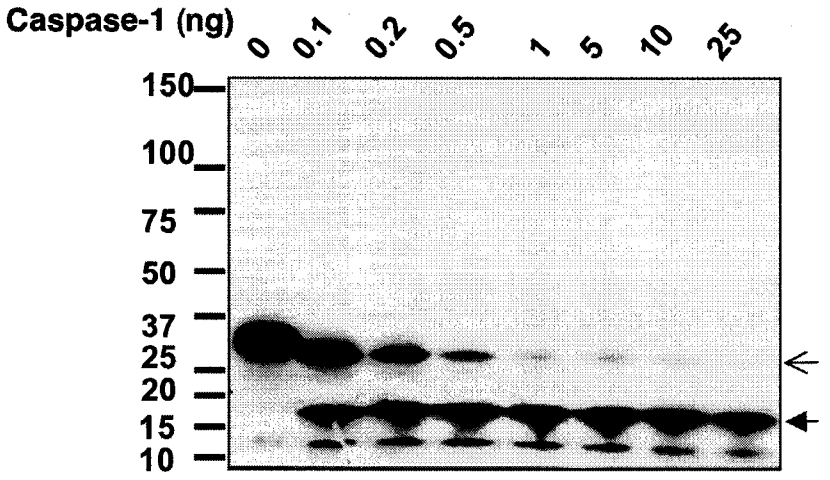
Figure 14

C



GAPDH

D



Pro- IL-1

Figure 14 Calibrating cleavage of ITT Glycolysis Enzymes by Caspase-1

in vitro transribed and translated ^{35}S labeled α - Enolase, aldolase, GAPDH, TIM and pyruvate kinase were incubated with or without recombinant caspase-1 of indicated amount for 4 hours at 37 ℃ , resolved in 4 – 12% SDS PAGE and viewed by autoradiography. Opened arrows indicate full-length proteins, closed arrows indicate cleaved fragments.

(A) α - Enolase ITT

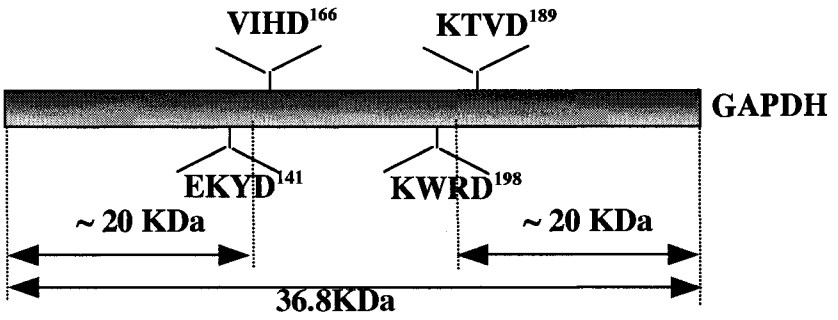
(B) Aldolase ITT

(C) GAPDH ITT

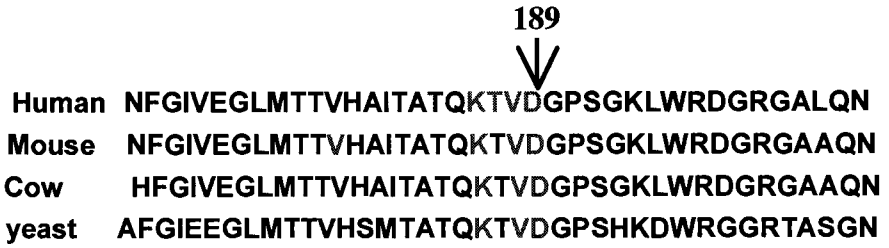
(D) Pro IL-1 β ITT

Figure 15

A



B



C

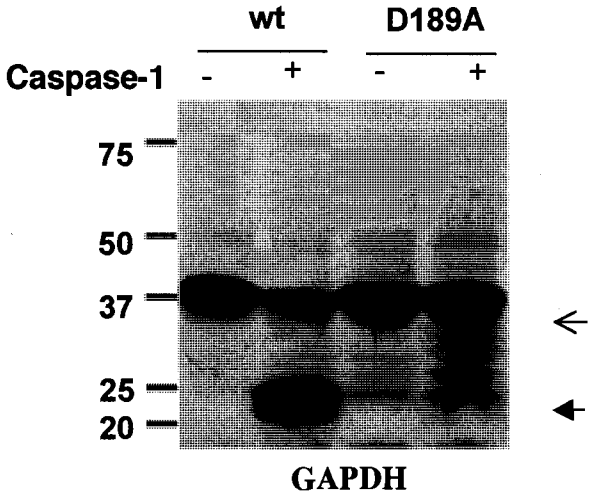


Figure 15 Identification of Caspase-1 Cleavage Site of in GAPDH

(A) Analysis of potential cleavage sites for GAPDH

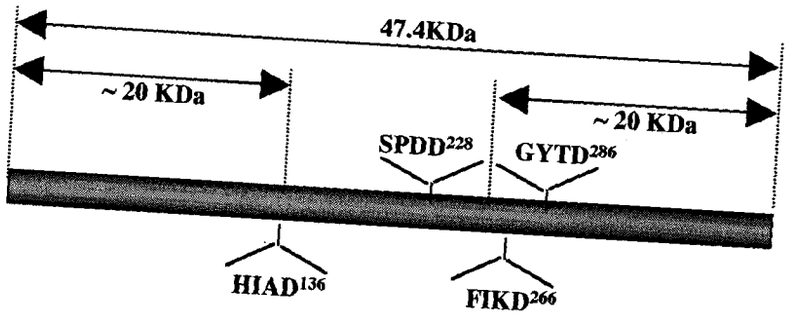
(B) Alignment of region containing caspase-1 cleavage site in GAPDH from different species

© Blockage of cleavage of GAPDH by caspase-1 in GAPDH $D^{189}A$ mutant

In vitro transcribed and translated 35^S labeled GAPDH and GAPDH D189A were incubated with or without 170 ng recombinant caspase-1 for 4 hours at 37 ℃ , resolved in 4 – 12% SDS PAGE and viewed by autoradiography. Opened arrows indicate full-length proteins, closed arrows indicate cleaved fragments.

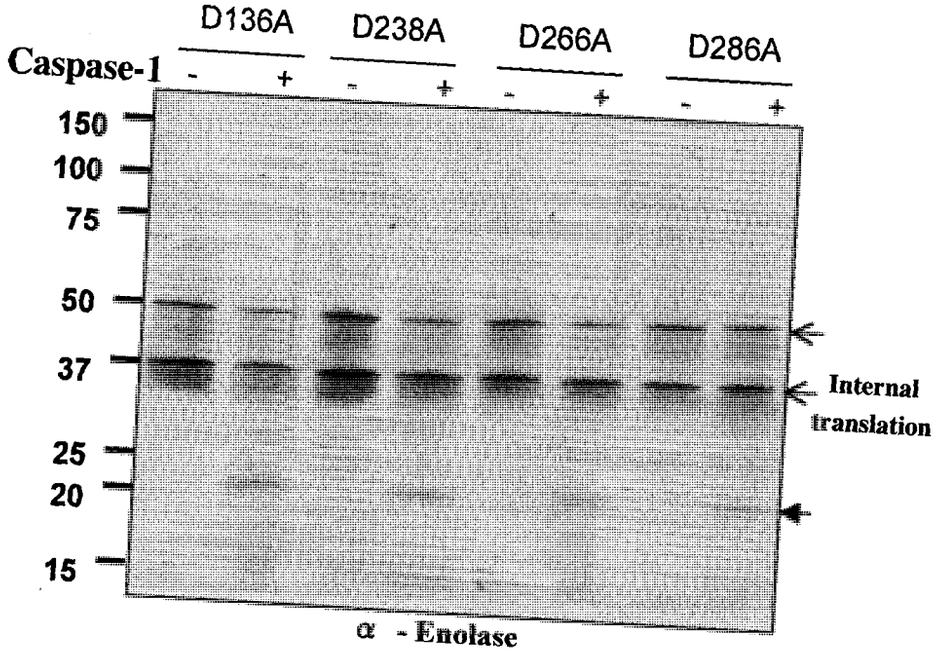
Figure 16

A



α - Enolase

B



α - Enolase

Figure 16 Identification of Caspase-1 Cleavage Site in α - Enolase

(A)Analyzing cleavage site for α - Enolase

(B)Failure to block cleavage by caspase-1 in 4 potential cleavage site mutants of α-Enolase

In vitro transcribed and translated 35^S labeled α - Enolase and its mutants were incubated with or without 170 ng recombinant caspase-1 for 4 hours at 37 ℃ , resolved in 4 – 12% SDS PAGE and viewed by autoradiography. Opened arrows indicate full-length proteins, closed arrows indicate cleaved fragments.

Figure 17

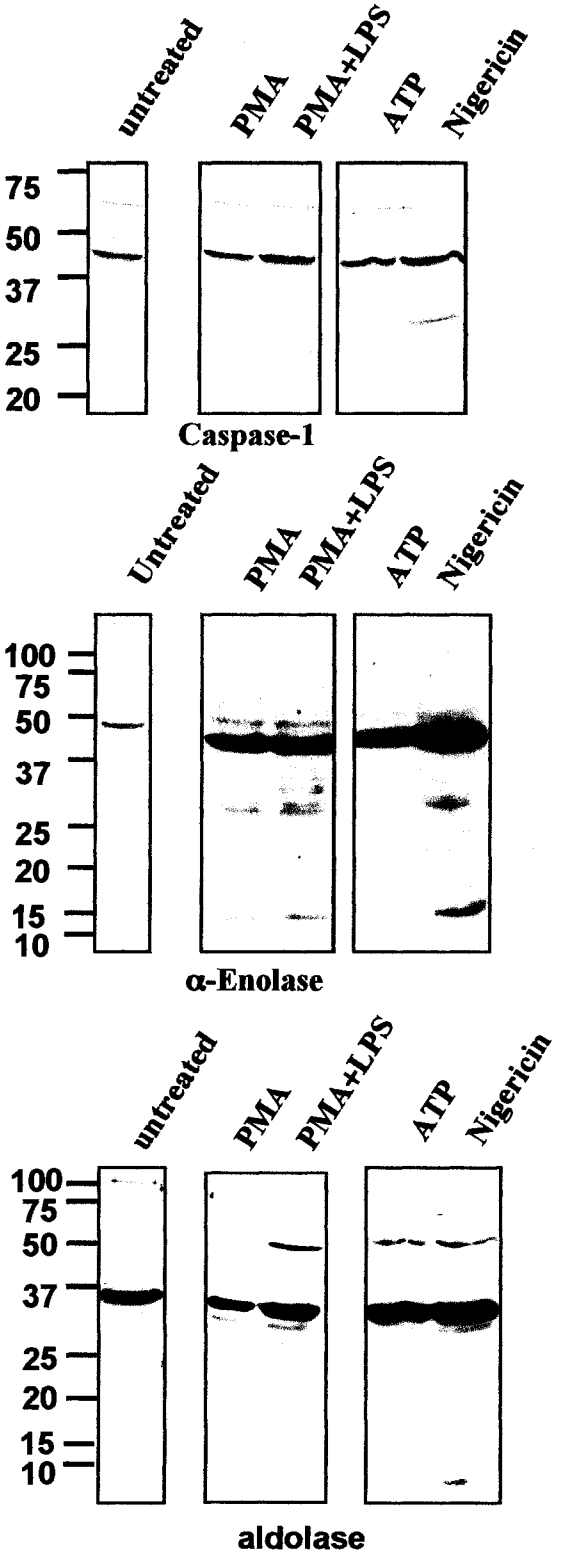

untreated
PMA
PMA+LPS
ATP
Nigericin
75
50
37
25
20
Caspase-1
Untreated
PMA
PMA+LPS
ATP
Nigericin
100
75
50
37
25
20
15
10
α-Enolase
untreated
PMA
PMA+LPS
ATP
Nigericin
100
75
50
37
25
20
15
10
aldolase

Figure 17 Western blot of Glycolysis Enzymese in ATP/Nigericin treated THP-1 Cells

THP-1 cells were pre-treated with PMA (20ng/ml) overnight and then treated with LPS (1μg/ml) for 3 hrs and further treated with ATP or nigericin to activate caspase-1. Cells were lysed in SDS loading buffer and resolved in 4-12 % SDS PAGE. Western blot was performed using antibodies against caspase-1, α - enolase and aldolase.

Figure 18

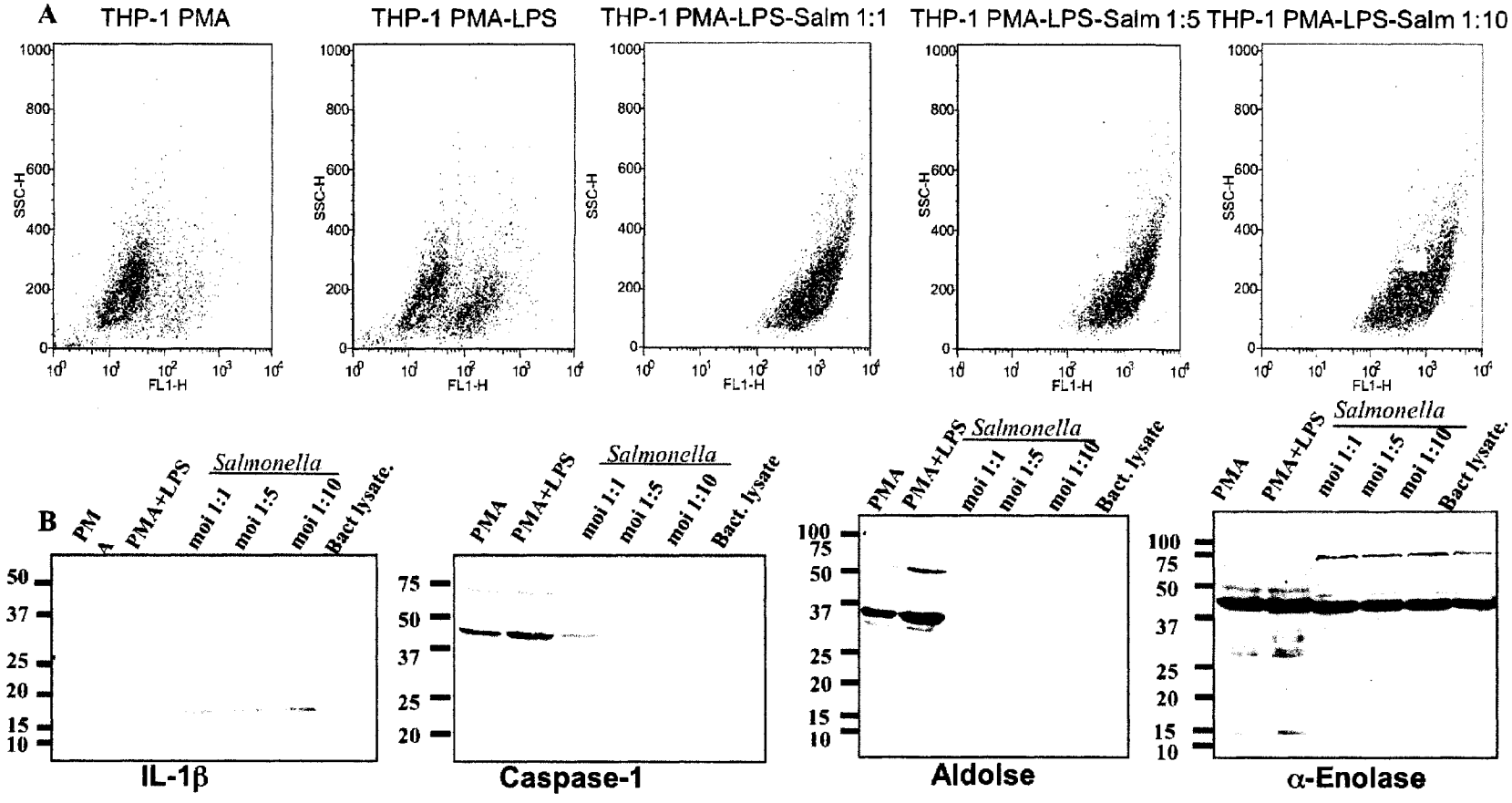

A
THP-1 PMA
THP-1 PMA-LPS
THP-1 PMA-LPS-Salm 1:1
THP-1 PMA-LPS-Salm 1:5
THP-1 PMA-LPS-Salm 1:10
SSC-H
FL1-H
B
PMA
PMA+LPS
Salmonella
moi 1:1
moi 1:5
moi 1:10
Bact lysate.
IL-1β
Caspase-1
Aldolse
α-Enolase

Figure 18 Depletion of Glycolysis Enzymes in *Salmonella* infected THP-1 cells

THP-1 cells were pre-treated with PMA and primed with LPS and then infected with *Salmonella*. Activation of caspase-1 was monitored by FACS analysis with FLICA^{casp-1}. Cells were lysed in SDS-PAGE buffer and lysates resolved on a 4-12% SDS PAGE. Western blot was performed using antibodies against caspase-1, mature IL- 1β, α-enolase, and aldolase.

(A) FACS with FLICAcasp-1

(B) Western Blot with antibodies against caspase-1, mature IL- 1β, α-enolase and aldolase.

Figure 19

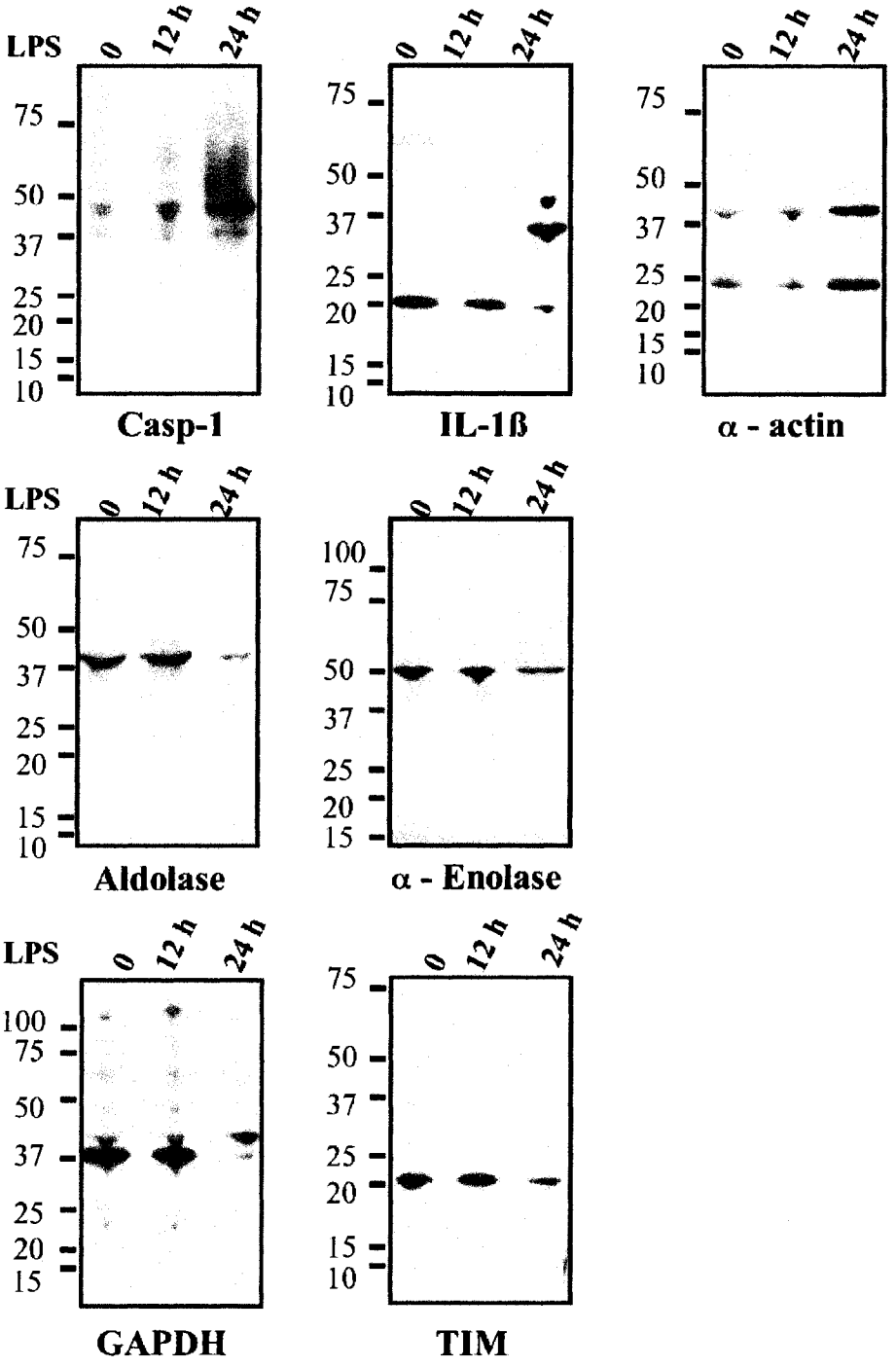

LPS
0
12 h
24 h
75
50
37
25
20
15
10
Casp-1
IL-1ß
α - actin
Aldolase
100
α - Enolase
GAPDH
TIM

Figure 19 Depletion of glycolysis Enzymes in Mice Muscle with Septic shock

Mice were injected intraperitoneally with 20mg/kg LPS to induce septic shock. The mice were killed 12 or 24 hrs post-injection. Diaphragm muscle was excised, and proteins were extracted and subjected in 4-12 SDS PAGE, western blot were performed using antibodies against caspase-1, IL-1ß, α-enolase, aldolase, GAPDH and actin.

Figure 20

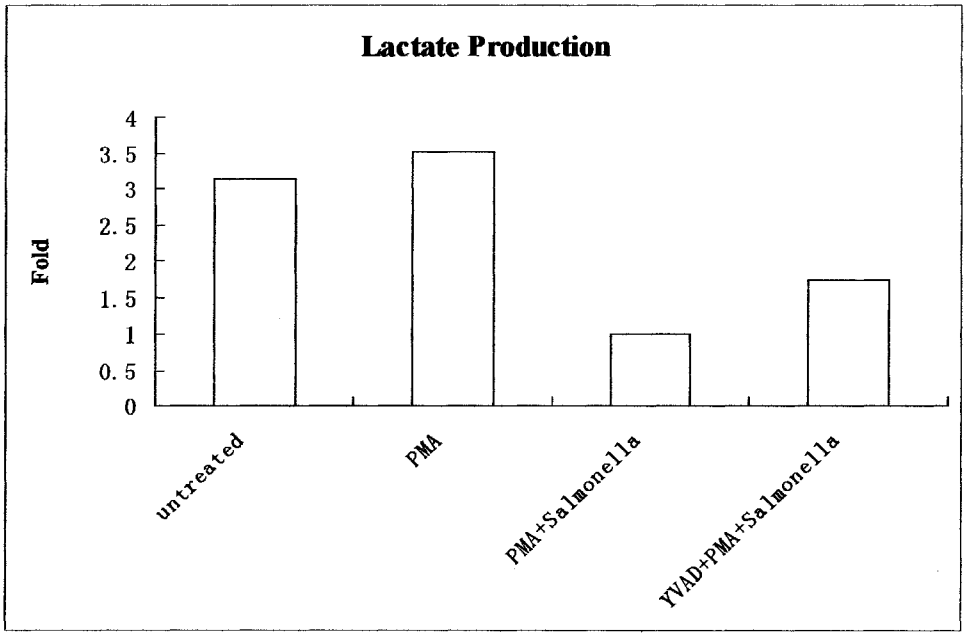


Figure 20 Change of Glycolysis Rate in THP-1 Cells After *Salmonella* Infection

THP-1 cells were pretreated with PMA(20 ng/ml) for 18 hrs, and infected with Salmonella (moi 1:10) for 4 hrs in the presence or absence of YVAD-FMK. Rate of glycolysis was assessed by Lactate production

Table 1 Caspase-1 and -3 substrates obtained by diagonal gel approach

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
Cytoskeletal and membrane proteins					
adenylyl cyclase-associated protein	gi|5453595	membrane protein	X	X	
actin-related protein 3	gi|5031573	membrane protein	X		
cellular myosin heavy chain	gi|553596	cytoskeletal protein		X	X
coronin-like protein	gi|1002923	cytoskeletal protein assembly/signaling	X		
cytokeratin 9	gi|435476	cytoskeletal protein	X		
F-actin capping protein α-1 subunit	gi|5453597	cytoskeletal protein	X	X	X
filamin 1	gi|4503745	cytoskeletal protein		X	X
gelsolin isoform a	gi|4504165	cytoskeletal protein		X	X
gelsolin isoform b	gi|38044288	cytoskeletal protein		X	X
keratin 1	gi|7331218	cytoskeletal protein	X		X
stomatin peptide	gi|181184	membrane protein		X	
tropomyosin isoform	gi|9508585	cytoskeletal protein		X	
vimentin	gi|37852	cytoskeletal protein		X	X

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
α-actin	gi|178027	cytoskeletal protein		X	X
α-tubulin	gi|15010550	cytoskeletal protein	X	X	X
β-tubulin	gi|1297274	cytoskeletal protein	X	X	X
γ-actin	gi|178045	cytoskeletal protein	X	X	X
Chaperons and proteins for folding					
Calnexin	gi|13097684	retains incorrectly folded glycoproteins in ER	X	X	
calreticulin precursor	gi|4757900	chaperon	X		
glutathione transferase	gi|87564	protein folding	X		
gp96 precursor	gi|15010550	heat shock protein, chaperone			
HSP27	gi|662841	heat shock protein, chaperone	X		
HSP60	gi|77702086	heat shock protein, chaperone		X	
HSP70 protein 8	gi|5729877	heat shock protein, chaperone	X	X	X

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
HSP90	gi|306891	heat shock protein, chaperone	X	X	X
protein disulfide isomerase-related protein 5	G i|1710248	protein folding	X		
PPIB precursor	gi|118090	Peptidyl-prolyl cis-trans isomerase B precursor, protein folding	X		
Transcription and nuclear proteins					
A+U-rich element RNA binding factor	gi|2547076	transcription	X		
APEX nuclease	gi|17939646	transcription	X		
H1 histone family, member 2	gi|4885375	structural protein of chromatin		X	
H1 histone family, member 3	gi|4885377	structural protein of chromatin		X	
H1b histone	gi|356168	structural protein of chromatin	X		

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
HMG-1	gi|968888	structural protein of chromatin	X		
hnRNP 2H9B	gi|7739437	transcription	X		
hnRNP A1	gi|133254	Heterogeneous nuclear ribonucleoprotein		X	X
hnRNP A2/B1	gi|4504447	Transcription	X	X	
hnRNP C1/C2	gi|62088634	transcription	X		
hnRNP D	gi|870743	Transcription	X	X	
hnRNP E2	gi|460773	Transcription	X	X	
hnRNP G	gi|542850	Transcription	X	X	
hnRNP H1/H2	gi|6065880	Transcription	X		
laminin-binding protein	gi|34234	nulear protein	X		
p105MCM	gi|1197636	replication	X		
MCM5	gi|1232079	replication	X	X	
RNH	gi|12653783	Ribonuclease/ angiogenin inhibitor	X		

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
U2 small nuclear ribonucleoprotein polypeptide A'	gi|18605961	transcription		X	X
Translation machinery					
EEF Tu	gi|704416	elongation factor Tu	X	X	
EEF1A1	gi|48735185	elongation factor-1-α	X	X	X
EEF1D	gi|38522	elongation factor-1-δ	X		
EF1a-like protein	gi|12006049	elongation factor 1 α	X		
EF2	gi|181969	elongation factor 2	X		
nascent-polypeptide-associated complex α	gi|5031931	translation	X	X	X
ribosomal protein L13	gi|15431297	translation	X		
ribosomal protein L18	gi|4506607	translation	X	X	
ribosomal protein L6	gi|21410970	translation	X		
ribosomal protein L7a	gi|4506661	translation	X		
ribosomal protein P0	gi|12654583	translation	X		
ribosomal protein S10	gi|55665989	translation	X		

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
ribosomal protein S3	gi|7765076	translation	X		
ribosomal protein S7	gi|337518	translation	X		
ribosomal protein S9	gi|550023	translation	X		
Immune proteins					
CAP37	gi|240869	cationic antimicrobial protein		X	
Coagulation factor XIII A chain precuror	gi|119720	blood coaguation		X	
HLA-I Cw-2 α	gi|231429	human leukocyte antigen		X	
HLA-I heavy chain	gi|1196444	human leukocyte antigen		X	
Glycolysis and Energy metabolism					
2-phosphopyruvate-hydratase α-enolase	gi|693933	glucose metabolism	X	X	
aldolase A	gi|28614	glycolysis	X		
ATP synthase, ATP synthase, H+ transporting, mitochondrial F1 complex, β subunit precursor	gi|32189394	energy production	X	X	

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
ATP synthase, H+ transporting, mitochondrial F1 complex, α subunit, isoform a precursor	gi|13111901	energy production	X	X	
carbonic anhydrase II	gi|179780	metabolism	X		
catalase	gi|179950	metabolism	X		
dihydrolipoamide dehydrogenase precursor	gi|181575	metabolism		X	
eosinophil preperoxidase	gi|31183	metabolism		X	
ferritin light subunit	gi|182516	iron storage	X		
Glutathione S-Transferase M2-3	gi|5822513	protein folding	X		
glyceraldehyde-3-phosphate dehydrogenase	gi|31645	glycolysis	X	X	
glyoxalase I	gi|5729842	glucose metabolism	X		
Horf6 A Chain B	gi|3318842	Peroxidase Enzyme	X		
lactoferrin precursor	gi|12083188	iron metabolism		X	
leucine aminopeptidase	gi|4335941	metabolism		X	
malate dehydrogenase (cytosolic)	gi|5174539	metabolism	X		
malate dehydrogenase (mitochondial)	gi|12804929	metabolism	X		
Myeloperoxidase	gi|494397	metabolism		X	
Myo-Inositol Monophosphatase Chain B	gi|2914661	metabolism	X		

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
NADH cytochrome b5 reductase	gi|553254	metabolism	X		
pyruvate kinase	gi|35505	glycolysis	X	X	
rho GDP dissociation inhibitor (GDI)	gi|36038	signaling	X		
similar to catalase	gi|4557014	metabolism		X	
transaldolase 1	gi|5803187	glucose metabolism		X	
Triosephosphate isomerase , glycolysis	gi|136066	glycolysis	X		
Tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta	gi|80477445	metabolism	X		
α-enolase	gi|4503571	glycolysis	X		
Channels, transporter and signaling					
Annexin Iii	gi|1421662	membrane protein		X	
adenylate kinase 2 isoform b	gi|7524346	signaling	X		
ADP-ribosylation factor 4	gi|4502205	signaling	X		
Annexin A2	gi|16306978	membrane protein	X	X	
caspase-1	gi|2914146	maturation of IL-1β and apoptosis	X		X

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
endoplasmic reticulum protein 29 isoform 1 precursor	gi|5803013	signaling	X		
Rho GDP dissociation inhibitor β	gi|56676393	signaling	X		
globin β	gi|26892090	O_2 transporter		X	
GTP binding protein	gi|4092054	signaling	X		
guanine nucleotide-binding regulatory protein α-inhibitory subunit	gi|183182	signaling	X		
migration-inducing gene 10 protein	gi|41350401	signaling	X	X	
p64 CLCP	gi|895845	chloride channel	X		
PA28 β	gi|2136005	proteasome activator	X		
polypyrimidine tract-binding protein 1 a	gi|4506243	signaling		X	X
PPPIA	gi|190281	protein phosphatase I α subunit, signaling	X	X	
proteasome α type, 8 isoform 1	gi|68303561	signaling	X		
protein PP4-X	gi|189617	Annexin A4, membrane protein	X	X	
Rab5	gi|642532	ras-related small GTP	X		

Protein	accession number	Function	Casp-1	Casp-3	Known substrate
		binding protein			
Ran-specific GTPase-activating protein	gi|542991	signaling	X		
serum albumin	gi|28592	steroid carrier		X	
SET translocation	gi|4506891	related to myeloid leukemia	X		
TRAP-1	gi|1082886	TNF receptor type 1 associated protein		X	
Others					
hypothetical protein LOC345651	gi|63055057			X	
PREDICTED: similar to POTE2A	gi|51460457			X	
unnamed protein product	gi|28590			X	

Table 2 Cloning primers for PCR

cDNA and clone site	Primers	
GAPDH (Xhol)	forward	5'-GCGGCTCGAGATGGGGAAGGTGAAGGTCGG-3'
GAPDH (EcoR1)	reverse	5'-GCGGGAATCCTTACTCCTTGGAGGCCATGTGGG-3'
α -enolase (HindIII)	forward	5'-GCGGAAGCTTATGTCTATTCTCAAGATCCATGCC-3'
α -enolase (NOTI)	reverse	5'-GCGGGCGGCCGCTTACTTGGCCAAGGGG-3'
aldolase (HindIII)	forward	5'-GGCCAAGCTTATGCCCTACCAATATCCAGC-3'
aldolase (NOTI)	reverse	5'-GCGGGCGGCCGCTTAATAGGCGTGGTTAGAGACG-3'
α -enolase nested (HindIII)	forward	5'-GCGGAAGCTTCGGACAGTATCTGTGGGTACC-3'
α -enolase nested (NOTI)	reverse	5'-GCGGGCGGCCGCCGAGCTGCCTGAGCTGACACG-3'
aldolase nested (HindIII)	forward	5'-GCGGAAGCTTGGGGTGCCTCAACCACACTCCG-3'
aldolase nested (NOTI)	reverse	5'-GCGGGCGGCCGCGCCCCGAGGAGGCGGCCTCC-3'
TIM (HINDIII)	forward	5'-GGCGAAGCTTATGGCGCCCTCCAGGAAGTTCTTCG-3'
TIM (NOTI)	reverse	5'-GGCGGCGGCCGCTCATTGTTTGGCATTGATGATGTCC-3'
Pyruvate kinase (NOTI)	forward	5'-GGCGGCGGCCGCATGTCGAAGCCCCATAGTGAAGCCGGG-3'
Pyruvate kinase (XhoI)	reverse	5'-GGCGCTCGAGTCACGGCACAGGAACAACACGCATGG-3'

Table 3 Primers for potential cleavage sites mutagenesis

cDNA and mutation site		Primers	
α -enolase	D136A	forward	5'-CCCCCTGTACCGCCACATCGCTGCCTTGGCTGGCAACTCTGAAGTCATCC-3'
α -enolase	D136A	reverse	5'-GGATGACTTCAGAGTTGCCAGCCAAGGCAGCGATGTGGCGGTACAGGGGG-3'
α -enolase	D238A	forward	5'-GGGAAAGCTGGCTACACTGCTAAGGTGGTCATCGG-3'
α -enolase	D238A	reverse	5'-CCGATGACCACCTTAGCAGTGTAGCCAGCTTTCCC3-3'
α -enolase	D266A	forward	5-'CTTCAAGTCTCCCGATGCCCCCAGCAGGTACATC-3'
α -enolase	D266A	reverse	5'-GATGTACCTGCTGGGGGCATCGGGAGACTTGAAG-3'
α -enolase	D286A	forward	5'-CAAGTCCTTCATCAAGGCCTACCCAGTGGTGTC-3'
α -enolase	D286A	reverse	5'-GACACCACTGGGTAGGCCTTGATGAAGGACTTG-3'
GAPDH	D144A	forward	5'-GGGTGTGAACCATGAGAAGTATGCCAACAGCCTCAAGATCATCAGC-3'
GAPDH	D144A	reverse	5'-GCTGATGATCTTGAGGCTGTTGGCATACTTCTCATGGTTCACACCC-3'
GAPDH	D166A	forward	5'-CCCTGGCCAAGGTCATCCATGCCAACTTTGGTATCGTGGAAGG-3'
GAPDH	D166A	reverse	5'-CCTTCCACGATACCAAAGTTGGCATGGATGACCTTGGCCAGGG-3'
GAPDH	D189A	forward	5'-CTGCCACCCAGAAGACTGTGGCTGGCCCCTCCGGGAAACTGTG-3'
GAPDH	D189A	reverse	5'-CACAGTTTCCCGGAGGGGCCAGCCACAGTCTTCTGGGTGGCAG-3'
GAPDH	D198A	forward	5'-CCCTCCGGGAAACTGTGGCGTGCTGGCCGCGGGGCTCTCCAGAAC-3'
GAPDH	D198A	reverse	5'-GTTCTGGAGAGCCCCGCGGCCAGCACGCCACAGTTTCCCGGAGGG-3'

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APPENDIX I Caspase-1 substrate: GATA-4

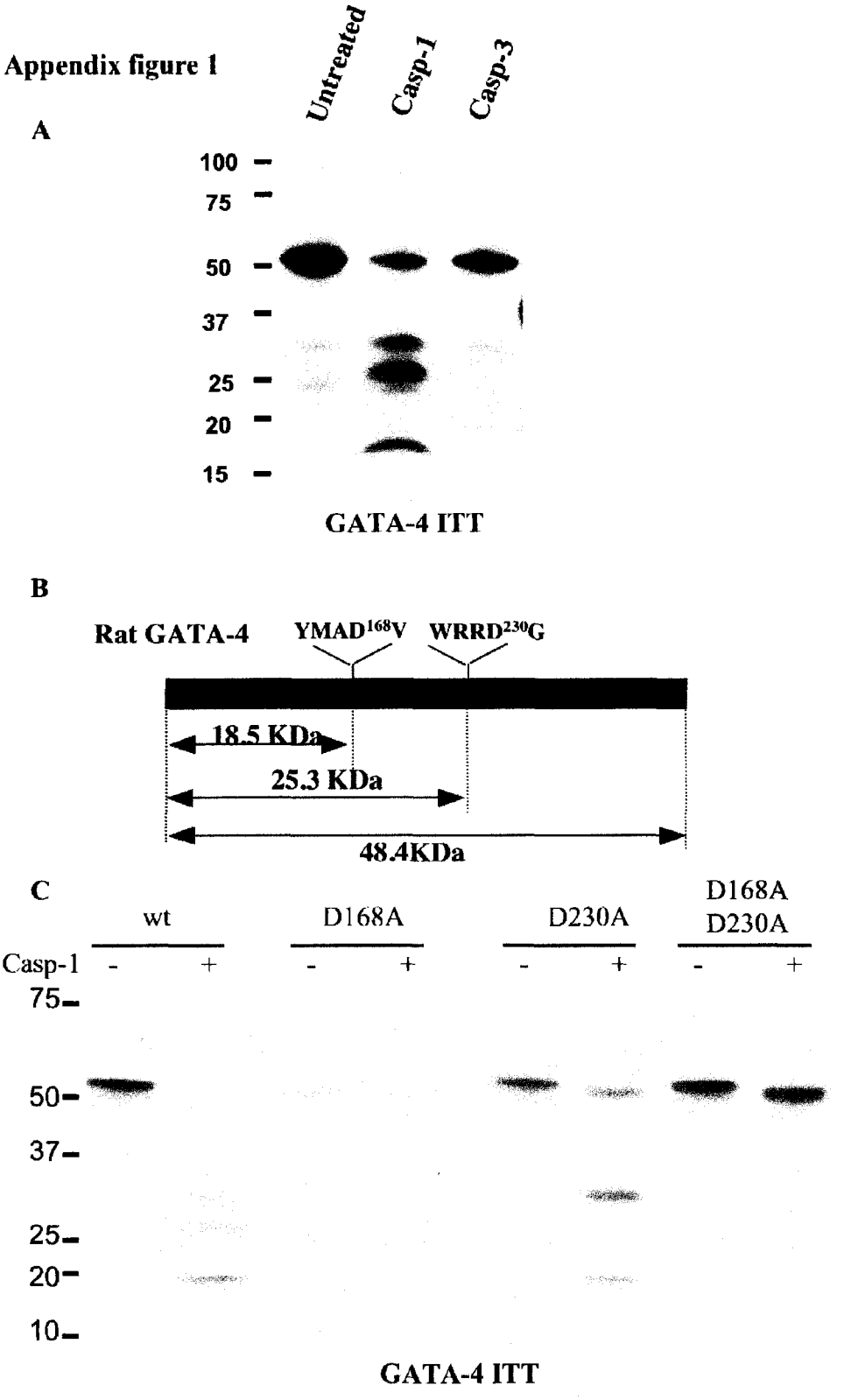
GATA-4 belongs to the GATA family of zinc finger transcription factors. It is expressed in developing cardiac cells and responsible for cardiac differentiation during early embryonic development (Heikinheimo et al., 1994). In adults, GATA-4 continues to be expressed in cardiac myocytes (Pikkarainen et al., 2004). It has been proposed that GATA-4 is required for the adaptive response of cardiomyocytes (Suzuki et al., 2004). GATA-4 was implicated in the protection of cardiac myocytes from apoptosis induced by antitumor treatment (Aries et al., 2004; Kim et al., 2003). The function of GATA-4 in cell survival was also supported by the fact that over expression of GATA-4 increased differentiation of cardiomyocytes, while inhibition of GATA-4 by an antisense strategy prevented cardiomyocyte differentiation and triggered extensive apoptosis (Grepin et al., 1997; Grepin et al., 1995). The action of GATA-4 in cell survival was suggested to occur through the the upregulation of Bcl-XL transcription by GATA-4 (Aries et al., 2004).

It has been shown that doxorubicin treatment induces apoptosis in cultured cardiomyocytes and GATA-4 is rapidly depleted during this process (Aries et al., 2004; Kim et al., 2003). To identify the factors that are responsible for the GATA-4 depletion during apoptosis, Dr. Mona Nemer' lab collaborated with our lab. Since there exist potential caspase-1 and –3 cleavage sites in the GATA-4 protein, we first tested the cleavage of GATA-4 by casapses-1, and –3. We *in vitro* transcribed and translated ^{35}S labeled rat GATA-4 and digested with recombinant caspase-1 and –3. Our results show that GATA-4 is cleaved by caspase-1 *in vitro*, but not by caspase-3 (Appendix I figure 1 A). Site-directed mutagenesis of potential cleavage sites of GATA-4 revealed that a combination of two point mutations D168A and D230A blocked caspase-1 cleavage (Appendix I figure 1 B, C). The cleavage sequences are YMAD^{168}↓V and WRRD^{230}↓G,

which fit the criteria of a bulky or hydrophobic amino acid in the P4 position of a caspase-1 substrate, and a small non-charged amino acid in the P1' position. Alignment of the GATA-4 sequences from different species showed that the cleavage site $WRRD^{230}\downarrow G$ is conserved in all the species examined, while the cleavage site $YMAD^{168}\downarrow V$ is conserved only in mammals (Appendix I figure 1 D). Structurally, GATA-4 contains an N-terminal and a possible C-terminal transcriptional activation domains, as well as two adjacent zinc fingers in the middle (Appendix I figure 2). Caspase-1 cleaves GATA-4 at $YMAD^{168}\downarrow V$ removing its N-terminal transcriptional activation domain and at $WRRD^{230}\downarrow G$ breaking one of its two zinc finger domains. Therefore, we hypothesize that the cleavage of GATA-4 by caspase-1 causes its inactivation.

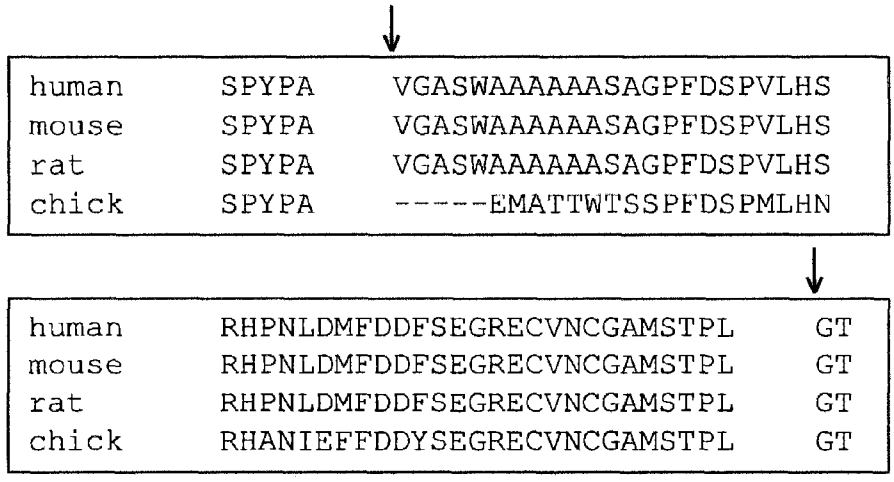
Dr. Nemer and her colleagues have observed that GATA-4 was rapidly degraded 3 hours after doxorubicin treatment of cultured cardiomyocytes (Aries et al., 2004). Cleavage products of GATA-4 were not detected by western blot. So, for future work, we need to confirm the *in vivo* cleavage of GATA-4 by caspase-1. Since it has been reported that some caspase substrates are degraded by the Ubiquitin/proteosome pathway (Demontis et al., 2006; Ditzel et al., 2003; Du et al., 2005), in a next step, we can test the possibility of GATA-4 degradation by this system. To do this, we would first inhibit the proteosome using the proteosome inhibitor LLnL, we would then expect the visualization of GATA-4 cleavage after Doxorubicin treatment. After the *in vivo* cleavage of GATA-4 by caspase-1 is confirmed, we will investigate the relevance of the GATA-4 cleavage on cell survival.

Appendix figure 1



Appendix figure 1

D



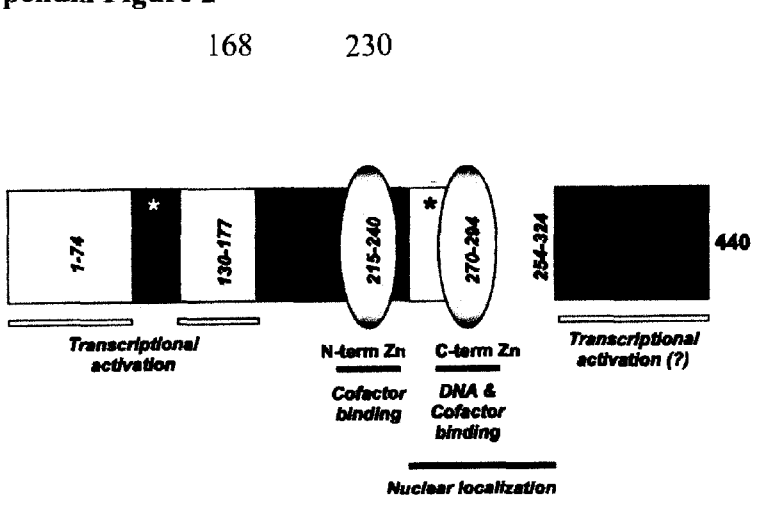
Appendix figure 1 Caspase-1 substrate GATA-4

(A) In vitro cleavage of ITT GATA-4 by caspase-1 and -3

(B) Analysis of GATA-4 cleavage sites

(C) Blockage of caspase-1 cleaving GATA-4 mutant (D168A D230A)

Appendix Figure 2



Appendix Figure 2 Functional Structure of GATA-4
(Adapted from Pikkarainen el., 2004)

GATA-4 contains an N-terminal and a possible C-terminal transcriptional activation domains, as well as two adjacent zinc fingers in the middle. Caspase-1 cleaves GATA-4 at $YMAD^{168}$ ↓ V removing its N-terminal transcriptional activation domain and at $WRRD^{230}$ ↓ G breaking one of its two zinc finger domains.

APPENDIX II