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Review

Caspase-2 redux

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Abstract

It has been difficult to assign caspase-2 to the effector or initiator caspase groups. It bears sequence homology to initiators (caspase-9 and CED-3), but its cleavage specificity is closer to the effectors (caspase-3 and -7). Interest in caspase-2 was dampened by the lack of a dramatic phenotype in the caspase-2 null mouse. Studies have been inhibited by the lack of knowledge about its mechanism of activation and the lack of specific methods to assay its activity. Molecular studies have defined a unique role for caspase-2 in apoptosis initiated by β-amyloid toxicity or by trophic factor deprivation. Recently, a role for caspase-2 as an upstream initiator of mitochondrial permeabilization has been proposed. Thus, while much remains to be deciphered about caspase-2, most critically the mode of activation, it is clear that caspase-2 plays critical and singular roles in the control of programmed cell death.
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Abbreviations: ARC, Apoptosis repressor with caspase recruitment domain; CARD, caspase recruitment domain; PACAP, proapoptotic caspase adaptor protein; RAIDD, receptor-interacting protein (RIP)-associated ICH-1/CED-3 homologous death protein with a death domain; TNF, tumor necrosis factor; TRADD, TNF-receptor-associated protein with death domain

Introduction

Although caspase-2 (NEDD-2, Ich-1) was the second mammalian caspase described,[1,2] it has not generated the sustained interest that has been focused on other members of the family such as caspase-3, -8 and -9. In the past year, this has changed a bit because of studies that have shown a role of caspase-2 in permeabilizing mitochondria.[3–7] In this review,

we will examine what is known about the role of caspase-2 and try to unravel the tangled functions ascribed to it.

Caspase-2 was initially described as a neuronally expressed caspase that was downregulated during the course of brain development.[1] Two forms of caspase-2 are found in mouse and man – a short antiapototic form and a longer proapoptotic form,[2] – although it is not clear that the shorter form is expressed as a protein. Caspase-2 is one of the most conserved caspases across species.[8,9] It shares sequence homology with the class of initiator caspases, especially ced3, caspase-9 and –1,[9] but its cleavage specificity is closer to the effector caspases, caspase-3 and -7.[10,11]

Thus, caspase-2 is a real orphan – it has several distant cousins but no known brothers or sisters. Its unique properties could indicate either that it has some unique functions or alternatively, that it is sufficiently unimportant that it does not need backup. The second view has been taken by many workers based on the lack of a dramatic phenotype in the caspase-2 null mouse. This animal appears quite normal apart from an increase in the number of oocytes.[12] In fact, several processes that had been demonstrated to depend on caspase-2 – such as the death of sympathetic neurons when NGF was withdrawn[13] and the sensitivity of lymphocytes to multiple stimuli, which induced caspase-2 cleavage[14,15] – were quite normal in these animals. These results, however, are misleading and have obscured very solid, if still somewhat confusing, work on caspase-2 function.

How is Caspase-2 Activated?

Before addressing the function of caspase-2, it is worthwhile to consider the existing data regarding the activation mechanism of caspase-2. When caspase-2 was identified there was one known mechanism of activation for caspases, sequential cleavage of the zymogen to release the large and small peptides that assembled to form the active heterotetramer. This mode of activation was assumed to hold for other caspases, and cleavage of caspase-2 was used as an indication of activation. Thus, much of the data showing a role for caspase-2 in a particular death paradigm relied on showing cleavage of caspase-2. Over the ensuing years, with the identification of at least 13 mammalian caspases, it has become clear that there are several ways of activating caspases. In addition to cleavage, these include oligomerization and formation of an apoptosome.[16–19] It is not clear yet as to which mechanism leads to activation of caspase-2. Identification of an adaptor protein for caspase-2, RAIDD, suggested that oligomerization was the mechanism of activation.[20] The initial report showed that RAIDD could interact with RIP and then with TNFR1 via TRADD and presumably recruit caspase-2 to this complex to carry out TNFR1-mediated death. However, it is not clear that RAIDD is always required for the activation of caspase-2. While the

overexpression of RAIDD can lead to cell death, blocking RAIDD or caspase-2 by dominant-negative forms did not abrogate TNF-mediated cell death, belying the relevance of RAIDD and/or caspase-2 in receptor-induced death. ARC is another protein that interacts with the caspase-2 CARD domain.[21] This was identified as an inhibitor of apoptosis but a subsequent study showed that it could also promote a caspase-2-dependent death.[22] ARC warrants further study, its function may be specific to a given cell type and/or stimulus. Among mammalian caspases, the prodomain of caspase-2 is most closely related in structure to that of caspase-9, both contain CARD-domains.[9] At present, caspase-9 is the only caspase known to be activated by apoptosome formation. In this mode of activation, cleavage of caspase-9 is not required for activation; in fact, the caspase-9 zymogen can have enzymatic activity and removal of the prodomain actually inactivates the enzyme, as it can no longer associate with the apoptosome.[23] Caspase-2 may also be activated in an apoptosome and thus the zymogen may have activity. PACAP (proapoptotic caspase adaptor protein), a novel proapoptotic protein that interacts with caspase-2 and -9, might be responsible for formation of a caspase-2 apoptosome.[24] Caspase-2 may not require an adaptor protein to oligomerize as it has been shown to homodimerize prior to cleavage, and overexpression of caspase-2 zymogen leads to apoptosis.[25] Certainly, more work needs to be done to determine the actual mechanism for caspase-2 activation. Studies show that the prodomain of caspase-2 is required for processing of caspase-2[25] and the cleavage sites in the zymogen have been identified.[26,27] But is active caspase-2 processed caspase-2? This is not yet known.

Bearing in mind that caspase-2 may not require cleavage to be activated, it is worth reconsidering the role of caspase-3 and/or caspase-9 in the activation of caspase-2. Multiple studies show that caspase-3 can cleave caspase-2 in vitro.[15,28,29] In various cell lines, multiple apoptotic stimuli, including staurosporine,[15] trophic factor deprivation,[30] etoposide[4,6,31] and gamma-irradiation[14] have been shown to lead to cleavage of caspase-2. Studies have shown that caspase-2 cleavage can be blocked by DEVD-CHO[15]. Cells lacking caspase-3 (MCF-7 cells), cells expressing a caspase-9dn construct, cells lacking APAF-1 or caspase-9[32] do not cleave caspase-2. These studies certainly show that cleavage of caspase-2, under certain conditions, is downstream of caspase-9/caspase-3, but at present it is not clear whether cleaved caspase-2 activated caspase-2. By analogy with caspase-9, removal of the prodomain from caspase-2 might actually inactivate the enzyme by preventing interaction with the apoptosome. Conversely, if cleavage is required for activation, the caspase-9 apoptosome may be acting to amplify death, a role recently ascribed to postmitochondrial caspases.[33] When expressed in *E. coli*, recombinant caspase-2 lacks the prodomain suggesting that proteolytically active caspase-2 might not require the prodomain. It is possible that caspase-2 has several active forms, including the zymogen, partially processed (i.e. cleavage of the p12 peptide) and fully processed. Without adequate measures of caspase-2 activity, as discussed below, this is difficult to resolve.

Measures of Caspase-2 Activity

This leads to the next question, what is an accurate measure of caspase-2 activity? Several studies have been done to identify specific substrates of individual caspases. These studies have grouped caspase-2 with caspase-3 and -7. A study using a combinatorial library found that all three had a preference for the substrate DEXD,[11] with the caveat that DEVD was cleaved effectively by caspase-3 and -7 but not cleaved at all by caspase-2. Another approach used a series of defined peptides and measured relative V_{max} and K_m values for the different caspases.[10] This study found that caspase-2 was unique in its requirement for a P5 residue and defined VDVAD as the optimal substrate and inhibitor of caspase-2. Unfortunately, the presence of a P5 residue does not prevent caspase-3 and -7 from efficiently cleaving this substrate as well.[10,34] This lack of specificity renders VDVAD-amc cleavage inadequate as a measure of caspase-2 activity and inhibition of death by VDVAD-FMK is not specific for caspase-2. Golgin-160 is a substrate of caspase-2, -3 and -7, with a unique caspase-2 cleavage site.[35] This may afford a measure of caspase-2 activity but this has not yet been widely used. Lack of knowledge of the activation mechanism and lack of a specific assay for caspase-2 activity have hampered the study of caspase-2. Also, the assumption of specificity using assays that are not specific (i.e. VDVAD assay) has resulted in the attribution of roles for caspase-2 in death pathways that may not be caspase-2 mediated.

Where is Caspase-2?

Studies on the subcellular localization of caspase-2 have done little to shed light on its function. Caspase-2 has been reported to reside in Golgi, mitochondria, nuclei and the cytoplasm. A recent study using multiple antibodies to different epitopes of caspase-2 finds expression in nuclei, cytosol and Golgi, without much evidence of expression in the mitochondria.[32] Another study showed that both caspase-2 and -9 were released from mitochondria after treatment of cells with Atr, a ligand of the adenosine nucleotide translocator.[36] These differences may be because of the variation with cell type as well as with the antisera used to detect caspase-2. Localization of caspase-2 in the Golgi[32,35] renders it unique among caspases in subcellular distribution. Golgin-160, the only known substrate of caspase-2 other than itself, resides in the Golgi and is cleaved under certain apoptotic conditions, such as staurosporine and UV-induced death. The caspase-2 cleavage of golgin-160 precedes disassembly of the Golgi and suggests that the Golgi complex may have a signal-transducing role during apoptosis. Caspase-2 also contains two nuclear import signals in its prodomain and overexpression of GFP-tagged caspase-2 results in nuclear localization.[5,37,38] When overexpressed, nuclear caspase-2 may be sufficient to induce mitochondrial permeabilization.[5] Purified caspase-2, in the absence of any cytosolic factors, has also been shown to cause mitochondrial permeabilization.[3,6]

Does Caspase-2 have a Function in Death Pathways?

A number of parameters have been used in attempts to determine whether an individual caspase is a critical

component of a death pathway. These include the detection of cleaved portions of the zymogen, direct detection of activity as seen by the cleavage of artificial substrates after exposure to the death stimulus and prior to morphologic signs of death. Other approaches have abrogated death by inhibition or removal of the specific caspase. A distinction must be made between caspase activation and caspase activity. Caspases can be activated but have their activity suppressed/inhibited by endogenous inhibitors of apoptosis (IAPs), as has been shown for caspase-3, -7 and -9. In the case of caspase-2, data about activation and activity are not obtainable and will not be until the mechanism of activation is delineated and a specific measure of activity devised. Inhibition by pharmacologic means is not specific enough, as discussed above. Therefore, we are for the moment limited to using molecular methods to remove or attenuate caspase-2 to obtain definitive information on its function. Table 1 shows the various death paradigms in which caspase-2 has been proposed to play a role. They are discussed in more detail below.

Trophic Factor Deprivation-mediated Death

Caspase-2 has been found to mediate trophic factor deprivation death in several different cell types, including Rat-1 cells,[2] PC12 cells[13,39] and primary cultures of sympathetic neurons.[13,40] In each of the cell types, trophic factor deprivation leads to the generation of an intermediate cleavage product of caspase-2. In addition, a variety of molecular approaches, including overexpression of the short form of caspase-2,[2] antisense oligonucleotide-mediated knockdown of caspase-

2[13,40] and expression of full-length antisense to caspase-2[39] have pointed to a critical role for caspase-2 under these conditions. These data were all thrown into question when it was reported that sympathetic neurons from caspase-2 null mice were not protected from trophic factor deprivation.[12] In fact, the lack of any dramatic phenotypic or developmental alterations in these mice raised the question of whether caspase-2 was absolutely necessary under any conditions. This apparent problem was resolved when it was shown that brains and sympathetic neurons from caspase-2 null animals had increased expression of both mRNA and protein for caspase-9 and the proapototic IAP inhibitor, DIABLO/Smac.[40] These compensations revealed parallel pathways for trophic factor death, one requiring caspase-2 and the other dependent on caspase-9. In neurons from wild-type animals, the caspase-9 pathway is suppressed by MIAP3 (XIAP). The choice of the pathway that executes death is determined by the relative expression of the components of positive and negative regulators of the pathway. The existence of parallel death pathways may speak of the importance of certain death pathways in certain biological processes. The development of the nervous system is very complex, involving the programmed death of many neuroblasts and neurons in an exquisitely timed fashion. Amazingly, this program is almost always carried out properly, yielding organisms with perfectly functioning nervous systems. Since proper execution of death pathways is so important in the developing nervous system, it would make sense that a system of fallback pathways has developed. In the case of caspase-2 null mice only the nervous system has been studied for changes in the

Table 1

Death stimulus	Cell type	Measure of caspase-2	Confirmed in caspase-2 null animals
Trophic factor deprivation	Rat-1 cells	Protection by overexpression of caspase-2 short[2]	
	Mo7E	Caspase-2 cleavage[14]	
	PC12 cells	Antisense knockdown[13,39,40]	
	Primary sympathetic neurons		Compensated by caspase-9 and Diablo upregulation
TNFα	HeLa	Caspase-2 cleavage[15]	No
	293	Overexpression RAIDD[20]	
	IMR90E1A	Cleavage to p33[29]	
	MDA	Overexpression casp2ProL[31]	
Cytokine deprivation	FDC-P1	Full-length antisense caspase-2 expression[48]	No
Staurosporine	Jurkat	Caspase-2 cleavage[15]	No
β-amyloid	Primary sympathetic neurons	Antisense oligonucleotide knockdown, Caspase-2 null neurons[41]	Yes
Anti-Fas	Jurkat	Caspase-2 cleavage[15]	No
	U293	Full-length antisense caspase-2 expression	
	CEM	Overexpression casp2ProL[42]	
Etoposide	Mo7E	Caspase-2 cleavage[14]	No
	Jurkat	VDVAD-ase activity VDVAD inhibition Full-length antisense caspase-2 expression[6] Overexpression casp2ProL	
	IMR90E1A	siRNA knockdown[4]	No
Ischemia	Rat hippocampus	VDVAD protection[43]	No
Camptothecin	Namalwa cells	Overexpression Casp2Lpro[31]	No
GTP-depletion	HIT-15	VDVAD-ase activity[49]	No
4-hydroxynonenal	RKO	VDVAD-ase activity[50]	No
Seizures	Rat	VDVAD-ase activity[51]	No

expression of caspases and caspase regulatory molecules. It would be quite interesting to see if these compensations are confined to the nervous system or if they are also found in systems that do not undergo developmental death. In the light of recent findings that some death paradigms utilize a pathway where bcl-2 regulates activation of initiator caspases upstream of the mitochondria,[33] another level of compensation may also occur in the caspase-2 null animals, a functional compensation for the lack of caspase-2 by another initiator caspase that would act upstream of the mitochondria, as shown in Figure 1.

β-Amyloid-induced Neuronal Death

One paradigm that is supported in both cell culture work as well as primary neurons from wild-type and caspase-2 null animals is β-amyloid-induced neuronal death. In this paradigm, the death pathway is conserved in central and
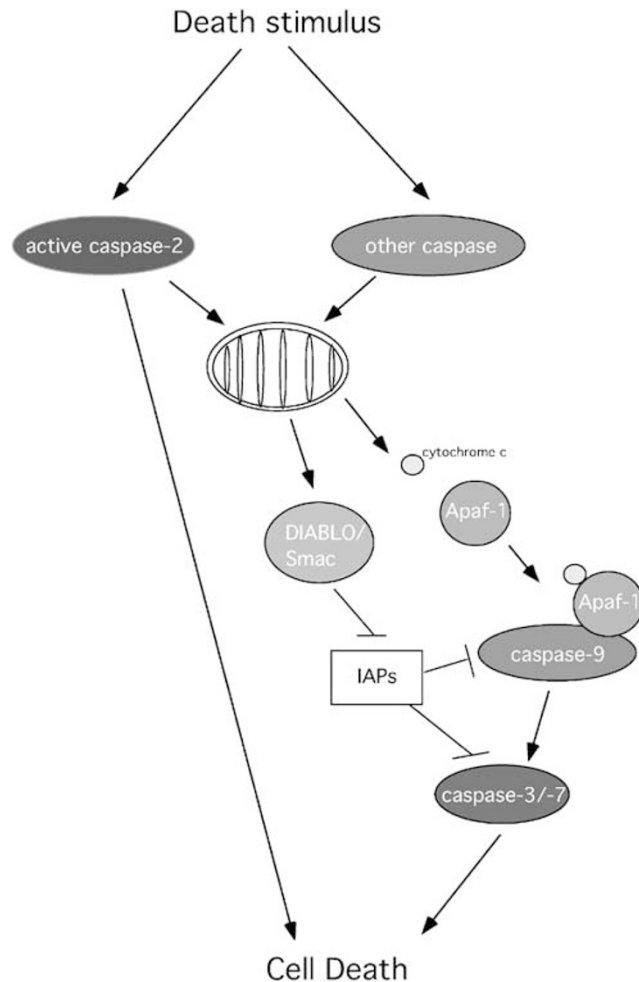
Figure 1 Model of caspase-2-mediated cell death. A specific death stimulus can activate caspase-2 leading to either a nonmitochondrial path to death or to permeabilization of mitochondria with release of cytochrome *c* and other proapoptotic factors. In the absence of caspase-2, under certain as yet undefined conditions, there may be activation of another initiator caspase leading to permeabilization of mitochondria. The relative contribution of each part of this pathway may be specific to both cell type and death stimulus

peripheral neurons and in the PC12 neuronal cell line.[41] Removal of caspase-2 acutely or chronically confers resistance to β-amyloid. It is curious that there are compensations in the caspase-2 null animal that have no effect on β-amyloid-induced death. Again, this may be attributable to the relevance of the death stimulus to neuronal development. Trophic factor deprivation plays a major role in sculpting the developing nervous system but it is unlikely that β-amyloid has much, if any, role in normal neuronal development.

Death-Receptor-mediated Death

TNFα-induced death

Studies linking caspase-2 to receptor-mediated death via activation of the TNFreceptor have been done in a variety of cell lines, including Jurkat, 293, IMR90E1A, MDA and HeLa cells.[20,29] The discovery of RAIDD provided an adaptor molecule that could recruit caspase-2 to the TNF death-inducing signaling complex.[20] However, interfering with RAIDD did not protect from TNF-α death and it was proposed that caspase-8 was also mediating death. Other evidence for caspase-2 involvement in TNF death included cleavage of caspase-2 to the p33 intermediate[15,29] and blockade of death by overexpression of Casp2LPro, a molecule proposed to be an endogenous inhibitor of caspase-2.[31] Caspase-2 null embryonic fibroblasts showed no protection from TNFα.[12] However, the data showing that both caspase-2 and -8 might be simultaneously activated would suggest that the caspase-2 null cells would not be protected.[20] What remains to be determined is which pathway is physiologically relevant. This may vary among types of cells, depending upon the relative expression of the components of the two death pathways, as seen with trophic factor deprivation death.

Fas-mediated death

A role for caspase-2 in another death-receptor paradigm has been suggested by the data from Jurkat, HeLa, U293, and CEM cells exposed to anti-Fas. Expression of full-length antisense to caspase-2 or expression of Casp2Lpro prevented release of cytochrome *c* and abrogated death.[42] Again, caspase-2 null lymphoblasts were not protected from Fas-mediated death.[12] A similar situation to that found for TNF may occur for Fas, that is induction of a caspase-8 pathway as well as a caspase-2 pathway.

Ischemic Neuronal Death

In a rat model of focal ischemia, there is an induction of the transcripts for caspase-2 long and short.[43] Infusion of VDVAD-FMK improved neuronal survival, but, as discussed above, this could be because of inhibition of caspase-3 or -7 or -2. There is also work showing a requirement for caspase-3 in ischemia.[44] The newer data do not conclusively show a function for caspase-2 in ischemic neuronal death. The lack of protection from ischemic damage in the caspase-2 null mice[12] suggest, that either caspase-2 is not relevant or that there are redundant or parallel death pathways in ischemia. Since the caspase-2 null brains show significant elevation in the

expression of caspase-9 and DIABLO/Smac[40] this pathway may be executing ischemic death in the caspase-2 null mice. The issue of transcriptional regulation of caspase-2 and its relation to caspase-2 function is an interesting one. Caspase-2 was initially identified as a neuronal developmentally down regulated gene but, of course, the caspase-2 null phenotype does not support a unique role for caspase-2 in neuronal development. Other death paradigms show transcriptional effects on caspase-2. Estrogen-withdrawal-induced death increased transcription of caspase-1 and −2,[45] but a causal connection between the transcriptional effects and function of caspase-2 has not yet been shown.

DNA Damage and Mitochondrial Permeabilization Death Pathways

The newest function proposed for caspase-2 is in the permeabilization of mitochondria, as mentioned in the beginning of this review. Four different groups have reported links between caspase-2 and release of mitochondrial pro-apoptotic factors during death. Death stimuli include etoposide[4,6] and overexpression of caspase-2[3,5] in either transformed cells or tumor-derived cell lines. One study utilized siRNA to specifically knockdown caspase-2 acutely,[4] introducing another specific, potent molecular tool for the study of death pathways. Overall, the data present compelling evidence for caspase-2 acting upstream of mitochondria. However, this phenomenon may be specific to these cells or to the apoptotic stimulus. Several agents inducing DNA damage, including etoposide, were found to induce death in lymphoblasts from caspase-2 null mice,[12] suggesting that caspase-2 may not be required for DNA damage death in lymphoblasts or that there is compensation by other caspases. Caspase-2 null lymphoblasts have not been examined for the relative expression of caspases and caspase regulators. Compensations as shown for brain may also occur in lymphoblasts, resulting in a lack of phenotype. Alternatively, there may be multiple caspases that can lead to mitochondrial permeabilization. A recent study presents a model for death of hematopoietic cells where bcl-2 controls the activation of initiator caspases upstream of the mitochondria.[33] In the proposed pathway apoptotic death occurs regardless of the presence of the caspase-9 apoptosome, the apoptosome acts as an amplification machine but is not critical for apoptotic death. Unlike earlier works that conclude that death in the absence of the apoptosome is caspase-independent and nonapoptotic,[46,47] this work shows clearly that death in the absence of either Apaf-1 or caspase-9 is apoptotic. DEVD-aomk is used to identify the active caspases, revealing activation of caspase-1 and -7 and another as yet unidentified caspase, possibly caspase-11 or -12. DEVD-aomk would probably not react with caspase-2, so it is possible that caspase-2 could also play a role here. The choice of initiator caspase used may depend on the relative abundance of the different caspases in a particular cell type or at a specific developmental stage.

Other Death Pathways

The other death mechanisms attributed to caspase-2, but not supported by the phenotype of the caspase-2 null mice (see Table 1), utilize non-neuronal cells. The studies using knock-down of caspase-2 all used cell lines, which were either transformed or derived from tumors. It is possible that these cells employ death pathways different from primary cell cultures. Generalization of conclusions reached on the basis of studies in cell lines to specific differentiated cells *in vivo* or *in vitro* must be done with caution. Another issue to be considered is the comparison of knockout animals, a chronic removal of a gene from the beginning of development, with acute knockdown of protein expression in a mature cell, whether by antisense or by siRNA. The response to removal of a gene and its product and the compensatory mechanisms utilized by the developing animals may be quite different from those of a mature cell. Knockout animals as well as cell culture systems are model systems with which to study cell death, and conclusions from each must be drawn with the inherent drawbacks of the model in mind. A better approach might be inducible knockouts where perhaps compensations would be less likely to occur. A double caspase-2/caspase-9 inducible knockout might resolve some of the questions about the function of these caspases relative to each other.

Conclusions

In summary, it appears that caspase-2 is the default executioner caspase, as well as initiator caspase, in neuronal cells that have been deprived of nerve growth factor and in neuronal cells that have been exposed to lethal doses of Aβ. In each of these cases it can be demonstrated that other caspases can replace caspase-2 as the executioner if inhibitors and facilitators of apoptosis are appropriately regulated (Figure 1). It is also clear that there exist many cell death paradigms where the executioner is not caspase-2, but rather caspase-3 or -7. Exciting recent studies have shown an upstream role for caspase-2 regulating the release of mitochondrial constituents. It is too soon to be certain that this 'upstream' role is confined to caspase-2 or whether a similar function might be served by other caspases. It is now critical to determine the nature of the signals that lead to caspase 2 activation and that focus its activity on the mitochondria. We need to understand the mechanism of caspase-2 activation when it is acting 'upstream' and to determine whether this is the same as the mechanism of activation when it is serving as an executioner. Many questions remain to be resolved about caspase-2.

It is clear that this 'orphan' plays a number of critical and singular roles in the control of programmed cell death in the organism and it is likely that its days as an 'orphan' living in relative seclusion have ended.

Acknowledgments

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