Le molecole effettrici della via apoptotica appartengono ad una famiglia di enzimi chiamati **CASPASI**:
Cisteine proteasi che scindono le proteine in corrispondenza di residui di aspartato.

**Substrati:**
- altre procaspasi
- lamina nucleare
- citoscheletro…

Cascata di attivazione
intracellular aspartic acid-directed proteases are synthesized as inactive precursors that are activated by dimerization or proteolytic processing.

Prodomain

![Proteolytic cleavage and Asp-X cleavage sites]

Active caspase dimer

Table 1: Structural and functional characteristics of cytosolic endopeptidases of the caspase family

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Map position of gene</th>
<th>Size of enzyme precursor (kDa)</th>
<th>Prodomain type</th>
<th>Active subunits (kDa)</th>
<th>Adapter protein</th>
<th>Caspase-activating complex</th>
<th>Caspase-proteolytic specificity</th>
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</thead>
<tbody>
<tr>
<td>Apoptotic initiator caspases</td>
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<td></td>
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<tr>
<td>Caspase-2</td>
<td>7q34-q35</td>
<td>31</td>
<td>Long, with CARD region</td>
<td>19/12</td>
<td>FADD, RIP1, FLIP, FLICE-like</td>
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<tr>
<td>Caspase-8</td>
<td>2q33-q34</td>
<td>35</td>
<td>Long, with two DD domains</td>
<td>18/11</td>
<td>FADD, DR5, DFFA, ASC</td>
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<tr>
<td>Caspase-9</td>
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<td>Apaf-1, Nav-1, Apoptosome</td>
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<tr>
<td>Caspase-10</td>
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<td>FADD, DEDAF, DISC</td>
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<td></td>
</tr>
<tr>
<td>Caspase-12</td>
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<td>Long, with CARD region</td>
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<td>TRAF-2</td>
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Apoptotic effector caspases

<table>
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<tr>
<th>Enzyme</th>
<th>Map position of gene</th>
<th>Size of enzyme precursor (kDa)</th>
<th>Prodomain type</th>
<th>Active subunits (kDa)</th>
<th>Adapter protein</th>
<th>Caspase-activating complex</th>
<th>Caspase-proteolytic specificity</th>
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<td>Caspase-7</td>
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<td>Short</td>
<td>20/12</td>
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</table>

Apoptotic caspases in mammals. The effector and initiator caspases are shown in red and purple, respectively. The position of the first intra-chain activation cleavage (between the large and small subunits, p20 and p10, respectively) is highlighted by a black arrow, whereas other sites of cleavage are represented by grey arrows. These other cleavage events are thought to modulate caspase activity and the regulation of caspases by inhibitor of apoptosis (IAP) proteins and by other proteins. Unlike other proteasezymogens, removal of the N-terminal prodomain of a caspase is unnecessary for its catalytic activity. The prodomains in initiator caspases invariably contain homotypic interaction motifs, such as the caspase-recruitment domain (CARD) and the death-effector domain (DED). The four surface loops (L1–L4) that shape the catalytic groove are indicated. The catalytic residue Cys is shown as a red line at the beginning of L2. The p20 and p10 subunits together form a caspase monomer. The caspases and the location of functional segments are drawn to scale.
Proteins of the inhibitor of apoptosis (IAP) family include XIAP (X-linked IAP), c-IAP1, c-IAP2, ILP2 (IAP-like protein-2), ML-IAP (melanoma IAP)/Livin, NAIP (neuronal apoptosis-inhibitory protein) and survivin, and are also known as MIA/HILP1, MIA/HILP2, MIA/HILP1, Ts-IAP, KIAA, BIRC1 and TIA1, respectively. A conserved linker peptide that precedes the BIR2 (baculoviral IAP repeat-2) domain of XIAP, c-IAP1 or c-IAP2 (shown in red) is responsible for inhibiting caspases-3 and -7 in mammals. On the basis of structural information, residues 128–143 (FLLINKOOGNILAKYDIR) of NAIP are predicted to carry out this function, as indicated in red. Only the BIR3 domain of XIAP can potently inhibit caspase-9. The biochemically characterized BIR domains that have known functions are highlighted in colour, whereas other domains in the various IAPs are shown in grey. CARD, caspase-recruitment domain.
at least two major pathways for caspase activation and apoptosis have been delineated, including an ‘extrinsic’ pathway triggered by the TNF family death receptors and an ‘intrinsic’ pathway activated by damage to mitochondria leading to cytochrome c release into the cytosol.

However, apoptotic stimuli do not often act through a single signaling pathway. In so-called ‘type II’ cells, the death receptor and the mitochondrial pathway are interconnected via caspase-8-mediated translocation of truncated Bid (tBid) to mitochondria, where tBid triggers cytochrome c release into the cytosol.
Raf-1 was originally described as the initiator of a mitogen-activated protein kinase (MAPK) cascade involved in the regulation of cell proliferation and differentiation (Davis, 1994; Robinson and Cobb, 1997).

Surprisingly, three independent Raf-1 knockouts in mice, generated with different gene targeting strategies, showed normal MAPK activation in embryonic fibroblasts (MEFs), suggesting that Raf-1 may be dispensable for MAPK signaling.

However, based on the increase in apoptosis in mutant embryonic tissues and the increased sensitivity of Raf-deficient mouse embryonic fibroblasts to apoptotic agents, it was concluded that the primary function of Raf-1 is to protect cells from apoptosis independently of the MAPK cascade.
Several mechanisms have been proposed to explain the antiapoptotic function of Raf-1. It has been suggested that Raf-1 may promote cell survival by antagonizing the proapoptotic apoptosis signal-regulating kinase 1 (ASK1) through a direct inhibitory interaction with the N-terminal domain of ASK1 (Chen et al., 2001).

It was also reported that Raf-1 translocates to the mitochondria in response to survival stimuli, where it mounts an antiapoptotic response independently of ERK activation (Wang et al., 1996; Salomoni et al., 1998; Peruzzi et al., 1999; Alavi et al., 2003). It was suggested that, when located to the mitochondria, Raf-1 suppresses cell death by inactivating the proapoptotic Bcl-2 family member Bad.
Apoptosis of hematopoietic cells induced by growth factor withdrawal is associated with caspase-9 mediated cleavage of Raf-1

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Here, we report that Raf-1 is rapidly cleaved by caspase-9 during apoptosis of hematopoietic cells induced by IL-3 withdrawal. Raf-1 processing cleaves off the N-terminal regulatory domain, leaving an intact C-terminal kinase domain (Raf-1-Ct) that is predominantly localized to the mitochondria. Furthermore, constitutive expression of mitochondria-targeted Raf-1-Ct significantly delays apoptosis of pre-B cells induced by IL-3 withdrawal. These results suggest an important role for caspase-9 mediated cleavage of Raf-1 as a negative feedback regulatory mechanism of hematopoietic cell apoptosis.

Raf-1 is efficiently cleaved in hematopoietic cells undergoing apoptosis through the mitochondrial death pathway

Structural and functional studies have shown that Raf-1 is composed of two distinct domains, an N-terminal regulatory domain (containing the conserved regions CR1 and CR2) and a C-terminal kinase domain (constituting the third conserved region, CR3). The amino-terminal domain suppresses the catalytic activity of Raf-1, and its deletion constitutively activates Raf-1.
IL-3 dependent pre-B cell line Ba/F3. Upon IL-3 withdrawal, Ba/F3 cells were arrested in the G1 phase of the cell cycle and died apoptotically, as demonstrated by DNA hypoploidy and proteolytic activation of caspase-3.

Ba/F3 cell lysates were prepared at various times after IL-3 withdrawal for immunoblot analysis with anti-Raf-1 antibodies raised against a peptide corresponding to the C-terminus of Raf-1. Within 12 h after IL-3 withdrawal, at which time 10% of the cells were hypoploid, a faint band of B40 kDa representing the C-terminal fragment of Raf-1 could be detected (Figure 1a).

Promyeloid FDCP-1 cells and puromycine (1 mg/ml) treated Ba/F3 cells

The cell death response and the kinetics of Raf-1-cleavage induced by puromycine were similar to those induced by growth factor withdrawal.
To determine if Raf-1 is also cleaved upon triggering of the extrinsic death pathway, its cleavage in death receptor-mediated apoptosis was examined in L929sAhFas and L929sAhFas.Bcl-2 cells.

**Figure 2** Cleavage of Raf-1 requires activation of the mitochondrial death pathway. (a) L929sAhFas cells and (b) L929sAhFas.Bcl-2 cells were stimulated with anti-Fas antibodies (100 ng/ml) for the indicated periods of time. Cellular proteins were separated by SDS–PAGE, and the proteolytic processing of Raf-1 and caspase-3 was detected by immunoblotting. Arrowheads indicate full-length Raf-1 and the 40 kDa cleavage product. Induction of apoptosis was determined by measuring the percentage of hyploid cells.

**Processing of Raf-1 during apoptosis induced by growth factor withdrawal is mediated by caspase-9**

Caspases that are proteolytically activated during apoptosis induced by IL-3 withdrawal in Ba/F3 cells (Figure 3a).
The kinetics of proteolytic activation of procaspases-3 and -7 were similar to those of pro-caspase-9. In contrast, caspase-8 was cleaved at a later stage.

Processing of Raf-1 in the presence of peptide caspase inhibitors. Cells were grown in the presence of IL-3 (lane 2) or deprived of IL-3 for 16 h in the absence (lane 1) or presence of 200 mMz-VAD.fmk (lane 3), Ac-DEVD.fmk (lane 4) or z-IETD.fmk (lane 5). Proteolytic processing of Raf-1 was detected by immunoblotting with polyclonal anti Raf-1 antibodies.

**caspase peptide inhibitors**

<table>
<thead>
<tr>
<th>caspase peptide inhibitors</th>
<th>IL-3-depl.</th>
<th>+</th>
<th>+</th>
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<th>+</th>
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</thead>
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<tr>
<td>z-VAD.fmk</td>
<td>+</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Ac-DEVD.fmk</td>
<td>+</td>
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<tr>
<td>z-IETD.fmk</td>
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</table>

Cells were grown in the presence of IL-3 (lane 2) or deprived of IL-3 for 16 h in the absence (lane 1) or presence of 200 mMz-VAD.fmk (lane 3), Ac-DEVD.fmk (lane 4) or z-IETD.fmk (lane 5). Proteolytic processing of Raf-1 was detected by immunoblotting with polyclonal anti Raf-1 antibodies.
Ba/F3 cells stably overexpressing Bcl-2 (Ba/F3-Bcl-2; lane 3) or CrmA (Ba/F3-CrmA; lane 4) were depleted of IL-3 for 16 h. Stable transfectants which express only the puromycin resistance gene (Ba/F3-puro) served as a control (lanes 1 and 2).

Taking into account the activation of caspase-9 in IL-3 deprived cells, its z-VAD.fmk sensitivity and its positioning downstream of the mitochondria, caspase-9 is a likely candidate for the Raf-1-cleaving caspase.
Figure 4 Cleavage of Raf-1 and Raf-1 mutants by caspase-9.

To determine which of these sites corresponds to the caspase-9 cleavage site, each Asp residue was mutated to Ala, and the in vitro sensitivity of the respective Raf-1 mutants to caspase-9-mediated proteolysis was analysed.

Note that the 40 kDa cleavage product runs slightly slower in the case of the Raf-1 mutants when compared to wild-type Raf-1, which is due to the presence of an E-tag peptide epitope (GAPVPYPDPLEPRAA) that was fused to the C-terminus of the Raf-1 mutants.
To investigate whether the D279A mutant is protected from cleavage in IL-3 deprived Ba/F3 cells, we stably expressed this mutant (Raf-1E-D279A) and the wild-type Raf protein (Raf-1E) in Ba/F3 cells and analysed their cleavage in IL-3-deprived transfectants (Figure 4d).

Whereas the apoptotic responses upon IL-3 withdrawal in the Ba/F3-Raf-1E and Ba/F3-Raf-1ED279A transfectants are comparable, as revealed by proteolytic activation of caspase-3, processing of the Raf-1E-D279A mutant is suppressed. This result suggests that the D279 caspase-9 cleavage site is also used in dying cells. However, some residual cleavage of the mutant could still be detected after 36 h of IL-3 deprivation, suggesting that in addition to the D279 cleavage site also (an)other cleavage site(s) (is) are (as for instance D273 or D337) maybe involved in Raf-1 processing.

To investigate whether the Raf-1-Ct fragment is still enzymatically active, we ectopically expressed Raf-1, Raf-1-Ct and their respective kinase defective (K375A) mutants in HEK293T cells and determined the phosphorylation state of the Erk1/2 kinase by immunoblotting with phospho-Erk-specific antibodies.
**Figure 5** The caspase-9 induced C-terminal fragment of Raf-1 shows increased activity compared to full-length Raf-1.

(a) Erk1/2 phosphorylation by Raf-1. Expression vectors encoding the wildtype Raf-1 kinase (Raf-1), the C-terminal cleavage product (Raf-1-Ct) and their kinase defective mutants (Raf-1 (K375A), Raf-1-Ct (K375A)) were transiently transfected in HEK293T cells.

(b) Induction of SRE dependent gene expression by Raf-1. The expression vectors encoding the wildtype Raf-1 kinase (Raf-1), the cleavage product (Raf-1-Ct) and their kinase defective mutants (Raf-1 (K375A), Raf-1-Ct (K375A)) were cotransfected with the pSRE-Luc plasmid (PathDetect, Stratagene), and with the pSV-Sport β-galactosidase plasmid. The latter was used to correct for variation of transfection efficiency. After 24 h, cells were lysed and the activities of luciferase and β-galactosidase were measured. Luciferase activity was normalized to the β-galactosidase activity. Bars represent the average (n=3)±s.d. of normalized luciferase activities. Bars are representative of two independent transfection experiments.

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**Figure 6** Raf-1-Ct is mainly localized to the mitochondria in IL-3 deprived Ba/F3 cells.

Immunoblots of cytosolic fractions (C) and mitochondrial fractions (M) of growing (ctrl) and IL-3 deprived (~IL-3) Ba/F3 cells (see ‘Material and methods’ for details). Equal amounts of protein were loaded in each lane (50 mg) and probed with antibodies against cytochrome oxidase subunit IV (COX IV), Erk1/2, or Raf-1.
Figure 7 Mitochondrial targeting of Raf-1-Ct enhances Ba/F3 cell survival in the absence of IL-3. Ba/F3 cells stably expressing a nontargeted or a mitochondria-targeted C-terminal Raf-1 fragment (Raf-1-Ct or M-Raf-1-Ct, respectively) or their kinase-defective mutants (Raf-1-Ct-K375A or M-Raf-1-Ct-K375A, respectively) were cultured without IL-3 for the indicated times.

(a) The percentage of hypoploid cells, determined by flow cytometry of propidium iodide-stained nuclei, is indicated as a measure for apoptosis. The mean values (7s.d.) of triplicate cultures are shown. (b) Cell extracts were assayed for caspase-3 activity based on the rate of Ac-DEVD-amc cleavage.

Cells transfected with Bcl-2 or only the puromycin resistance gene (Puro) were used as positive and negative control, respectively.

In conclusion, our results suggest an important role for caspase-9 mediated proteolytic activation of Raf-1 in the negative feedback regulation of hematopoietic cell apoptosis induced by growth factor depletion. In certain conditions, for instance during temporary but potentially lethal lack of survival factors, relief of Raf-1 autoinhibition by caspase-9 mediated release of the Raf-1 N-terminal regulatory domain might help to overcome the critical period by blocking the proapoptotic signal at the mitochondria. Refinement of this model remains an area for further study.