Regulation of Fas-mediated apoptotic signaling by MAPK signaling modules

Leon Tourian Jr.

Division of Experimental Medicine

McGill University
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Abstract

The activation of death receptor Fas (CD95/APO1) triggers apoptosis inducing caspase activation by direct (extrinsic) and mitochondria dependent (intrinsic) pathways. Mitogen activated protein kinases (MAPK), which include p38, JNK and ERK kinases, are also activated during Fas signaling and are known to either positively or negatively regulate apoptosis. Chemical inhibition of JNK (SP600125) and p38 (PD169316) sensitize tumor cells to Fas mediated apoptosis. I studied Fas mediated apoptosis in Jurkat cells and in some experiments HeLa cells. PD169316 is less potent than SP600125 and attenuates the effect of the later when present together. PD169316 inhibits two isoforms of p38 which either promote (p38α) or inhibit (p38β) apoptosis; I investigated their relative regulatory influences on Fas signaling. I show that p38α is essential for Fas mediated caspase-8 activation: it promotes the dephosphorylation and exclusion of c-FLIP$_S$ but not c-FLIP$_L$ from the death inducing signal complex (DISC). Distally both p38 isoforms positively influenced the intrinsic pathway by common and selective effects on pro-apoptotic Bcl-2 proteins. The sensitizing effects of p38 and JNK inhibition were ablated by Bcl-2 localized at the endoplasmic reticulum. In HeLa cells, the proapoptotic effects of p38α are overridden by the anti-apoptotic effects of ERK.

Résumé

Activation des caspases et l'induction de l'apoptose induient par Fas (CD95/APO1) requirent les voies de signalisation apoptotique intrinsèque et extrinsèque. Les enzymes « mitogen-activated protein kinases » (MAPK) influencent ces voies de signalisation soit positivement ou négativement. L'inhibition chimique de JNK (SP600125) a sensibilisé l'apoptose signalée par Fas à un degré plus important que la sensibilisation induite par l'inhibition chimique de p38 (PD169316) dans les cellules Jurkat et HeLa. Par contre, l'inhibition de p38 a atténué la sensibilisation induite lors de l'inhibition de JNK. J'ai analysé le rôle des deux isoforms de p38 connues pour soit promouvoir (p38α) ou inhiber (p38β) l'apoptose. Je démontre que p38α est essentielle pour l'activation de la caspase-8 activée par Fas. p38α promouvait la déphosphorylation et l'exclusion de c-FLIP$_S$ mais pas de c-FLIP$_L$ de la « death inducing signal complex » (DISC). Les deux isoforms de p38 promouvaient l'apoptose au niveau de la voie apoptotic intrinsèque via leur effet commun et sélectif sur les protéines Bcl-2. Bcl-2 localisée au niveau du réticulum endoplasmique bloque la sensibilisation négociée par l'inhibition de p38 et JNK. Dans les cellules HeLa, les effets proapoptotic de p38α sont dominés par les effets antiapoptotic de ERK.

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1. Introduction

1.1 Apoptosis

There are two mechanisms by which a cell can die: necrosis or apoptosis. Apoptosis is a programmed cell death event in which the dying cell plays an active role in its own elimination. Apoptosis is essential for maintaining organellar homeostasis in multicellular organisms in such diverse and fundamental processes as limb development (Zakeri and Ahuja 1994; Nagy, Thomazy et al. 1998) and elimination of infected or neoplastic cells (McCarthy 2003; Brown and Attardi 2005). Apoptosis is distinct from necrotic cell death which induces disruption of cell and tissue matrix. Necrosis occurs during inflammation induced by infection or injury.

1.1.1 Apoptosis occurs in four phases

The apoptotic process occurs in four distinct steps (Hacker and Vaux 1997). The first part is called the induction phase. During this initial step, death inducing and survival signals impinging on a cell are integrated. If death signals are dominant, molecular systems that trigger apoptosis are activated. Key regulators of the induction and the subsequent effector phase include the Bcl-2 and the mitogen activated protein kinase (MAPK) family of proteins. The second stage is the effector phase, which involves the irrevocable commitement of the cell to die. This segment of the apoptotic pathway is mediated by the mitochondria which becomes permeable and releases soluble pro-apoptotic factors into the cytosol promoting the activation of the third phase called the degradative phase. This phase is characterized by the activation of enzyme systems called

caspases responsible for triggering the biochemical and structural features of apoptosis. The last segment of the apoptotic process is known as the phagocytic phase which involves the engulfment of cell fragments produced during the apoptotic process by macrophages.

1.1.2 Inducers of apoptosis

Apoptosis can be generally initiated by four distinct stimuli (Wertz and Hanley 1996). The first class of apoptotic activators are cell surface receptors such as Fas (CD95/APO1), tumor necrosis factor receptor (TNFR) and TRAIL (all members of the death receptor superfamily) (Wallach, Varfolomeev et al. 1999), and a subtype of the somatostatin (SST) family of receptors (SSTR3) (Srikant 1995; Sharma, Patel et al. 1996). Upon binding to their cognate ligands, these receptors trigger a signal transduction cascade, which leads to caspase activation, increased mitochondrial membrane permeability and apoptosis. Intracellular assault is the second family of apoptotic stimuli, these include free radicals, anoxia, high intracellular free calcium levels and activation of perforin/granzyme B (Wolf, Schuler et al. 1999). These agents initiate apoptosis by directly increasing mitochondrial membrane permeabilty. The third class of apoptotic inducers is DNA damage induced by a wide variety of agents such as irradiation or anti-tumor chemotherapeutic drugs. The last apoptotic prompt involves direct cell membrane damage by extracellular aggressors such as irradation or free radicals (Wajant 2003).

1.1.3 The extrinsic and intrinsic apoptotic pathways

All four apoptotic triggers have a common denominator: the mitochondria. Direct insult to the mitochondria activates "the intrinsic apoptotic pathway" also known as "type II apoptotic signaling pathway". The intrinsic pathway is regulated by the Bcl-2 family of proteins which can either positively (via pro-apoptotic Bcl-2 family of proteins) or negatively (via anti-apoptotic Bcl-2 family of proteins) regulate apoptosis (Sprick and Walczak 2004).

Cell surface receptors capable of activating the caspase cascade induce apoptosis via a distinct route labeled "the extrinsic apoptotic pathway" also known as "type I apoptotic signaling pathway". The efficacy of this pathway is dependent on the expression levels of death receptors and their presence at the cell surface, the relative abundance of pro- and anti-apoptotic adaptor molecules bound to the receptor and the relative abundance of caspases activated by the receptor.

1.2 The caspase family of proteins

All four apoptotic prompts induce apoptosis by turning on caspases (Creagh, Conroy et al. 2003). Caspases are cysteine-aspartate specific proteases involved in triggering apoptosis. The protease family of caspases is grouped into two different subgroups: the initiator and effector caspases. Initiator caspases include caspase-2, -8, -9 and -10; these proteases are typically activated early on during an apoptotic cascade. Of these, caspase-8/FLICE is the most studied. Initiator caspases are responsible for cleaving and activating effector caspases. Effector caspases include caspase-3, -6 and -7: these caspases are responsible for mediating the degradation phase of apoptosis. Caspase-3 is by far the most studied and pivotal protease involved in inducing apoptosis in the effector subfamily (Takahashi 1999).

1.2.1 Caspase-induced biochemical and morphological changes

Activated caspases induce appropriate biochemical and structural changes to ensure that the dying cell is properly degraded and packaged to be eliminated by phagocytosis. The degradation process involves breakdown of cytoskeletal proteins located at cell surface and within the nucleus, cleavage of anti-apoptotic proteins and DNA, and alterations of the phospholipid constitution of the cell membrane (Bennett, Gibson et al. 1995). Cleavage of structural proteins induces cellular and nuclear shrinkage. This is followed by fragmentation of the cell into smaller fragments called apoptotic bodies. Apoptotic bodies are "mini cells" surrounded by a functional cell membrane with altered lipid composition. This alteration is due to the translocation of phosphotidylserine (PS) from the inner leaflet of the lipid bilayer to the outer leaflet and serves as a signal for the apoptotic cell to be phagocytosed by macrophages (Dini, Ruzittu et al. 1996).

Apoptosis also induces alterations of intracellular organelles. The most studied organelles are the mitochondria and the endoplasmic reticulum (ER) (Ferri and Kroemer 2001). These organelles display characteristic changes during apoptosis. Death signals induce increased mitochondrial membrane permeability due to pore formation within the mitochondrial membrane causing a dissipation of the inner mitochondrial transmembrane potential ($\Delta\psi_M$)) and release of soluble mitochondrial intramembrane proteins that regulate downstream apoptotic events (Bernardi, Scorrano et al. 1999). Apoptotic stimuli also induce loss of ER membrane integrity and subsequent leakage of calcium ions from the ER lumen into the cytoplasm. High intracellular calcium levels then activate the intrinsic apoptotic pathway (Rizzuto, Pinton et al. 2003).

1.3 The Bcl-2 family of proteins

The intrinsic apoptotic signaling pathway is regulated by the Bcl-2 family of proteins. All members possess at least one of four conserved motifs known as Bcl-2 homology (BH) domains (Kelekar and Thompson 1998). The anti-apoptotic members contain all four BH domains and include Bcl-2, Bcl-X_L, Bcl-w, A1 and Mcl-1. The pro-apoptotic members are separated into two classes: the Bax and the "BH3 only" families. The Bax family shares three BH domains (BH1, 2 and 3) and includes Bax, Bak, and Bok. The "BH3 only" family retains only the BH3 domain and includes Bim, Bik, Bad, Bmf, Bid, Noxa and Puma (Adams and Cory 1998; Adams and Cory 2001).

1.3.1 Anti-apoptotic Bcl-2 family members

The anti-apoptotic Bcl-2 members that inhibit apoptosis contain a hydrophobic carboxy-terminal domain which allows them to insert into the membranes of the mitochondria, the ER and the nuclear envelope (Nguyen, Millar et al. 1993). The most studied member of this family is Bcl-2. Bcl-2 is constitutively tethered on surface of the mitochondria, ER and nucleus (Zhu, Cowie et al. 1996). Bcl-X_L and Bcl-w are cytosolic proteins, however upon induction of a death signal they insert into specific intracellular organelles to inhibit apoptosis (Gonzalez-Garcia, Perez-Ballestero et al. 1994). Each member of the anti-apoptotic Bcl-2 family of proteins can single handedly inhibit apoptosis. At the level of the mitochondria, Bcl-2 is involved in maintaining mitochondrial membrane integrity and inhibits the actions of pro-apoptotic Bcl-2 members (Reed

1998). At the level of the ER, Bcl-2 is believed to be a key mediator in maintaining cellular calcium homeostasis as well as Ca2+-independent apoptotic events (Rizzuto, Pinton et al. 2003). The ER is the main calcium storage organelle of the cell allowing the maintenance of low calcium levels in the cytosol. Stress signals impinge upon the ER, induce loss of membrane integrity causing leakage of calcium into the cytoplasm; increased cytosolic calcium has been shown to be an important activator of apoptosis.

1.3.2 The pro-apoptotic Bax superfamily

Members of the pro-apoptotic Bax family of proteins such as Bax and Bak are essential for the activation of the intrinsic apoptotic pathway by facilitating the loss of $\Delta\psi m$ (Brady and Gil-Gomez 1998). Activation of the intrinsic pathway requires the presence of at least one member of Bax family; the absence of Bax and Bak inhibits the intrinsic pathway. Bax is a cytosolic protein, which in the presence of apoptotic signal translocates to the mitochondria and ER whereas Bak is constitutively present at the surface of these two organelles (Nechushtan, Smith et al. 2001). Apoptotic stimuli signal Bax and Bak to oligomerize, form pores within the mitochondrial membrane, increase mitochondrial membrane permeability and release soluble intramembrane pro-apoptotic proteins such as cytochrome c into the cytosol (Degenhardt, Sundararajan et al. 2002). The exact mechanism involved in the release of proteins from within the mitochondria remains unclear and highly debated. In cells induced to undergo apoptosis, Bax has also been shown to relocate to the ER and oligomerize with Bak to form pores through which ER pool of calcium is leaked into the cytosol (Zong, Li et al. 2003).

1.3.3 The pro-apoptotic "BH3 only" subfamily

The second class of proapoptotic proteins contain a sole BH3 domain. "BH3 only" pro-apoptotic members induce apoptosis by inhibiting anti-apoptotic Bcl-2 proteins. Bid is the most studied member of this family (Bouillet and Strasser 2002). Bid is cleaved by caspase-8 and the product of this cleavage (tBid) translocates to the mitochondria and potentiates the pro-apoptotic function of the Bax and Bak by facilitating Bax mediated pore formation within the mitochondrial membrane (Luo, Budihardjo et al. 1998). BH3-only proteins cannot induce apoptosis in the absence of Bax and Bak and therefore are considered to function upstream of Bax and Bak mediated apoptosis (Wei, Zong et al. 2001).

1.3.4 Post-translational modification of Bcl-2 family members

Bcl-2 action is modulated via phosphorylation (Haldar, Jena et al. 1995). To date there is no consensus on how phosphorylation modulates Bcl-2 anti-apoptotic function (Ling, Tornos et al. 1998). Some reports indicate that phosphorylation of Bcl-2 inhibits its anti-apoptotic action (Haldar, Jena et al. 1995) while others suggest that phosphorylation promotes its protective function (Ito, Deng et al. 1997). For example, phosphorylation of Bcl-2 at serine residue 70 restrains Bcl-2's anti-apoptotic effect and enables the efficient translocation of Bax to the mitochondria (Shitashige, Toi et al. 2001).

The pro-apoptotic actions of Bad are also regulated by phosphorylation. There are many Ser residues in Bad including those at 112, 136 and 155 which are phosphorylated. Bad

phosphorylation has been shown to promote cell survival (Downward 1999). Phosphorylation of Bad is mediated via many kinases such as Akt, Rsk, PAK, $P70^{S6K}$ and PKA (Datta, Dudek et al. 1997; Fang, Yu et al. 1999; Scheid, Schubert et al. 1999; Virdee, Parone et al. 2000; Zhou, Liu et al. 2000; Jin, Mao et al. 2002) which are activated by growth and survival factors. Removal of growth factors induce a dephosphorylation of Bad and allow it to bind to Bcl-2 and thus allows for the oligomerization of Bax and Bak and induces mitochondrial dysfunction leading to the release of pro-apoptotic proteins from within the mitochondria (Yang, Zha et al. 1995; Zhou, Liu et al. 2000). There are also reports suggesting that underphosphorylated Bad only interacts with pro-apoptotic members and not with Bcl-2 (Bae, Hsu et al. 2001). Phosphorylation of serine 136 has been shown to enable binding of adaptor molecule 14-3-3 blocking the pro-apoptotic function of Bad (Zha, Harada et al. 1996). Bad has been shown to be located within the cytosol and or tethered to the mitochondria in an inactive form especially when phosphorylated at serine 112 via a mitochondria-anchored protein kinase A (Harada, Becknell et al. 1999).

Bax (Gardai, Hildeman et al. 2004; Xin and Deng 2005) and Bid (Desagher, Osen-Sand et al. 2001) have also been shown to be phosphorylated; however how phosphorylation affects their pro-apoptotic function remains unclear.

1.4 Death Receptors

Death receptors represent a subgroup of the TNF receptor super family that initiates the apoptotic process. The death receptor family includes: Tumor Necrosis Factor Receptor 1 (TNFR1), CD95 (Fas/Apo-1), TNF-Receptor Related Apoptosis Mediated Protein (TRAMP/DR3/Apo-3), TNF-

Related Apoptosis-Inducing Ligand (TRAIL) Receptor-1 and -2 and DR6 (Wajant 2003). These death receptors all contain a death domain (DD) within their cytoplasmic tail. Upon ligation of these DD containing receptors by their respective ligands (Boldin, Varfolomeev et al. 1995; Hofmann and Tschopp 1995), there is recruitment of adaptor molecules responsible for relaying the death signal from the cell surface to intracellular effectors of apoptosis such as caspases, members of the Bcl-2 family of proteins and the MAPK family of enzymes.

1.4.1 Fas

CD95 (Fas/Apo-1) is a 45kDa Type I transmembrane protein (single α helix spanning the lipid bilayer) (Orlinick, Vaishnaw et al. 1999). The extracellular domain of CD95 is composed of 3 cysteine-rich repeats that serve as recognition sites for either Fas ligand (FasL) or a specific agonistic Fas antibody (Fas-mAb) (Nagata and Golstein 1995). In the inactive state or in the absence of bound ligand, Fas forms inactive complexes via its pre-ligand-binding assembly domain (Chan 2000). These inactive complexes are typically formed by the assembly of three Fas molecules (Holler, Tardivel et al. 2003). FasL or anti-Fas antibody recognize these inactive complexes and upon binding induce receptor oligomerization and the subsequent assembly of the death inducing signal complex (DISC) (Kischkel, Hellbardt et al. 1995). Formation of the DISC is required for Fas mediated apoptosis (Peter and Krammer 2003) .

1.4.2 Death inducing signal complex (DISC) assembly

Fas forms the DISC complex along with Fas-associated death domain (FADD) and FADD like interleukin-1b converting enzyme (FLICE) better known as procaspase-8. FADD is an adaptor molecule which contains a DD (Chinnaiyan, O'Rourke et al. 1995; Muzio, Chinnaiyan et al. 1996; Jeong, Bang et al. 1999). Fas/FADD interaction is coordinated thought the DD motifs found on both proteins. It is important to note that in the absence of FADD, Fas mediated apoptosis cannot proceed (Wajant, Johannes et al. 1998; Zhang, Cado et al. 1998). The FADD/caspase interaction is coordinated via a death effector domain (DED) present on both these proteins. Upon binding to FADD, procaspase-8 is clustered in close proximity to other procaspase-8 molecules enabling their autoproteolytic cleavage. Autoproteolytic cleavage results in the activation and release of the active form of these caspases into cytoplasm. The recruitment and binding mechanism of FADD to Fas appear to be dependent on its phosphorylaton status (Kennedy and Budd 1998; Rochat-Steiner, Becker et al. 2000; Scaffidi, Volkland et al. 2000; Alappat, Volkland et al. 2003; Shimada, Matsuyoshi et al. 2004; Zhang, Zhang et al. 2004).

Cellular- FLICE-inhibitory protein (c-FLIP) also binds within the DISC (Tschopp, Irmler et al. 1998). c-FLIP is expressed in either long (c-$FLIP_L$) or short (c-$FLIP_S$) form both of which contain two DEDs, allowing them to bind FADD and prevent procaspase-8 from joining the DISC and being activated. c-$FLIP_L$ contains an additional inactive caspase catalytic domain (Shu, Halpin et al. 1997; Rasper, Vaillancourt et al. 1998; Hu, Johnson et al. 2000; Yeh, Itie et al. 2000). Over-expression of either form of c-FLIP inhibits Fas mediated cell death (Scaffidi, Schmitz et al. 1999). The DEDs of both c-FLIPs allows them to bind either procaspase-8 and or FADD. Whereas c-$FLIP_S$ completely inhibits procaspase-8 cleavage, c-$FLIP_L$ permits its partial cleavage into a p43 form (Chang, Xing et al. 2002; Kataoka and Tschopp 2004; Kataoka 2005).

The regulatory mechanism involved in regulating c-FLIP binding and exerting its anti-apoptotic action remains unclear, however there is increasing evidence suggesting that post-translational modifications such phosphorylation are involved (Yang, Xiao et al. 2003).

1.4.3 Fas mediated activation of the intrinsic pathway.

Upon stimulation of Fas, both intrinsic and extrinsic apoptotic pathways become active (Depraetere and Golstein 1997; Wallach, Varfolomeev et al. 1999; Holmstrom and Eriksson 2000; Wajant 2002). The function of each pathway will depend on the intensity of caspase-8 activation and its ability to induce high levels of caspase-3 activity. If caspase-8 activation is robust, the extrinsic pathway will prevail. In this situation, the activated intrinsic pathway will serve to amplify the death signal by activating mitochondria resident caspase-9, which will increase caspase-3 activation (Knight, Riffkin et al. 2004). However, if caspase-8 activation is low, the intrinsic pathway will serve as the principal driving force of the apoptotic signal. Consequently cells have been classified Type I characterized by having high caspase-8 activity to directly process sufficient levels of caspase-3 or Type II exemplified by predominant activation of intrinsic pathway (Barnhart, Alappat et al. 2003).

1.5 Mitogen activated protein kinases and Fas signaled apoptosis

1.5.1 Introduction to MAPK pathways

The mitogen activated protein kinase (MAPK) family of proteins is the most studied group of kinases activated by Fas. The MAPK superfamily is comprised of three distinct signaling pathways: the p38 family of proteins, the c-jun-N-terminal kinases or stress activated kinases (JNK/SAPK) and the extracellular-signal-regulated protein kinase (ERK). Each of these three pathways operates in a three tier system (Wada and Penninger 2004). MAPK is a serine/threonine kinase that is activated by MAPK kinase (MAPKK). MAPKK is a dual specific kinase that phosphorylates at both Ser (serine)/Thr (threonine) and Tyr (tyrosine) residues. MAPKK phosphorylate MAPK on Thr-X-Tyr motif (Lawler, Fleming et al. 1998). The X is a glutamate, proline or glycine for ERK, JNK and p38 respectively. MAPKs are inactive unless phosphorylated. MAPKK are in turn activated by MAPKK kinases (MAPKKK). These kinases receive signals from wide variety of kinases. Activated MAPK are known to phosphorylate a number of substrates. The most important group of MAPK substrates are transcription factors, however MAPKs also phosphorylate members of the Bcl-2 family of proteins and cell cycle regulators.

1.5.2 p38

p38 MAPK is typically activated by environmental stresses including heat, osmotic and oxidative stresses, inflammatory cytokines and TNF receptor signaling (Johnson and Lapadat 2002). Activation of p38 has been shown to induce many cellular responses such as cell motility, apoptosis, chromatin remodeling, proliferation, differentiation, survival and osmoregulation (Ambrosino and Nebreda 2001). The MAPKK MKK3 and or MKK6 activate p38 MAPK. These are in turn activated by the MAPKKK: MEKKs, MLK and ASK1. There are

5 isoforms of p38 that been characterized to date: p38α, p38β, p38γ, p38δ and p38-2 (Eckert, Efimova et al. 2003). These isoforms differ in their substrate specificity and expression. p38 α and β are ubiquitously expressed and responsible for the activation of heat shock proteins 25 and 27 (HSP25/HSP27) and the AP-2 MAPK. p38-2, p38δ and p38γ are required for the activation of ATF-2. p38γ is expressed in skeletal muscle, heat, lung, testis and thymus while p38δ is present in lung, pancreas, small intestine, kidney, testis and epidermis (Hale, Trollinger et al. 1999).

The involvement of p38 during apoptosis was first elucidated using pyridylimidazole compounds such as SB203580 and PD169316 (McIntyre, Ponticello et al. 2002). Chemical inhibition has revealed the involvement of p38 in regulating the cleavage of Bid and the translocation of Bax to the mitochondria and ER (Zhuang, Demirs et al. 2000). However all existing synthetic inhibitors of p38 target the p38α and p38β isoforms, masking the specific and common actions of each isoform. While it is clear that a great number of apoptotic stimuli stimulate the activation of p38, its functional relevance is a debatable issue given the limitation of using chemical inhibitors. However, use of inhibitors has allowed the elucidation of a few roles of p38. There is increasing data indicating that p38α positively regulates apoptosis (Harrington, Smeglin et al. 2000; Zhang, Shan et al. 2003; Cao, Semenova et al. 2004; Ren, Zhang et al. 2005) while p38β plays an anti-apoptotic role during death receptor mediated apoptosis (Jiang, Chen et al. 1996; Nemoto, Xiang et al. 1998; Guo, Kang et al. 2001). However the mechanism of action of these two isoforms, and their specific regulatory input during extrinsic and intrinsic apoptotic signaling pathways remain unclear. Inhibition of p38α has also been shown to induce increased ERK activation during inflammation; this data indicates that inhibition of p38 dependent apoptosis induces proliferation via the ERK pathway (Xiao, Malcolm et al. 2002; Ohashi, Nakagawa et al.

2004). The anti-apoptotic role of p38 has been further elucidated with the observation that activated p38 is responsible for the phosphorylation and subsequent inhibition of caspase-8 and caspase-3 (Alvarado-Kristensson, Melander et al. 2004). The regulatory input during apoptosis of other p38 isoforms remains unknown; however they appear to be involved in non-apoptotic processes such as differentiation and development.

1.5.3 c-jun-N-terminal protein kinase (JNK)

The JNK subfamily is typically activated in response to a wide variety of stress signals such as heat shock, osmotic stress, pro-inflammatory cytokines, TNF receptors, ischemia and UV exposure (Davis 1994; Davis 2000). Activation of JNK has been shown to induce many cellular responses such as apoptosis, survival, development, inflammation and tumourigenisis. MAPKK MKK4 and MKK6 activate JNK. These in turn are activated by MEKKs 1-4 and ASK. JNK exists as three different forms: JNK1, JNK2 and JNK3. JNK1 and JNK2 (46 and 54kDa respectively) are ubiquitously expressed while JNK3 is a brain specific protein kinase. JNK1 and JNK2 activate transcription factors such as c-jun, ATF-2, Elk-1, Myc, p53 and MADD (Franzoso, Zazzeroni et al. 2003). Moreover JNK1 and JNK2 are also involved in the phosphorylation of many other proteins such as members of the Bcl-2 family of proteins (Bcl-2, Bcl-XL, Bad, Bax, and Bim) and cell cycle regulators such as p21 (Lin and Dibling 2002; Lei and Davis 2003; Lin 2003).

JNK1 and JNK2 are known to be activated by Fas and have been reported to have pro- and anti-apoptotic roles (Lin 2003) or no role at all (Tournier, Hess et al. 2000). There is also evidence

suggesting an anti-apoptotic role for these kinases when activated early on in a death cascade but a pro-apoptotic role later on during the death cascade (Krilleke, Ucur et al. 2003). Increasing evidence also suggests that JNK1 and JNK2 isoforms have opposite influence on apoptosis. JNK1, but not JNK2, appears to play a pro-apoptotic role (Chen, Meyer et al. 1996; Sun, Liu et al. 1998; Kuan, Yang et al. 1999; Park, Kim et al. 2000; Wang, Hua et al. 2000; Hochedlinger, Wagner et al. 2002). The exact role JNK1/2 play during apoptosis remains unclear. Some studies also suggest that these kinases are required for the release of pro-apoptotic proteins from the mitochondria. Genetic studies show that JNK1 and JNK2 are both anti-apoptotic kinases. For example, in mice lacking the JNK1 and JNK2 genes, ($jnk1^{-/-}$ $jnk2^{-/-}$ mice) apoptosis was increased in the hindbrain and forebrain regions (Kuan, Yang et al. 1999). Immature thymocytes, and peripheral mature T cells deficient in MKK, an upstream JNK activator, appear to be highly susceptible to Fas mediated cell death (Rincon, Whitmarsh et al. 1998). This effect was also observed in $jnk1^{-/-}jnk2^{-/-}$ in mouse embryonic fibroblasts (MEF). It is important to note that both JNK and p38 are involved in mediating Fas expression, while the latter kinase is also involved in the expression of FasL (Mansouri, Ridgway et al. 2003).

1.5.4 Extracellular-signal regulated protein kinase (ERK)

ERK MAPK is typically activated upon growth factor and mitogen stimulation. Upon activation these kinases stimulate transcriptional responses in the nucleus inducing proliferation, development, differentiation and or cellular survival (Johnson and Lapadat 2002). ERK exists as two isoforms: ERK1 (44kDa) and ERK2 (42kDa). ERK1/2 are activated by MEK1/2 while the latter are in turn activated by the MAPKKK c-raf which itself is activated by Ras. ERKs are

responsible for the phosphorylation of many substrates such as p90RSK S6, MAPK-activated protein kinase-1, MAPK-activated phosphatase, phospholispase-1 and MSK. The transcription factors which are activated by ERKs include Elk-1, Ets 1, Sap1a and m-Myc. ERKs are also known to phosphorylate STAT3, Sos, EGF and estrogen receptors (Rubinfeld and Seger 2004).

Given their predominant role in promoting cell survival and proliferation ERKs are generally considered as being anti-apoptotic. The ERK pathway has been shown to be involved in the phosphorylation and deactivation of the pro-apoptotic Bcl-2 member Bad (Jin, Mao et al. 2002). ERK1/2 have also been shown to phosphorylate caspase-9 and inhibit the intrinsic (Type II) apoptotic signaling pathway (Allan, Morrice et al. 2003).

1.6 Summary

MAPKs play a diverse role in a wide variety of cellular processes. ERK exerts an anti-apoptotic influence while p38 and JNK are either pro- or anti-apoptotic. The specific mechanism utilized by these kinases to regulate apoptosis is being gradually unveiled. ERK has been shown to inhibit apoptosis by phosphorylating both Bad and caspase-9 thereby blocking their pro-apoptotic function. P38 and JNK on the other hand have been shown to either inhibit or promote apoptosis. The discrepancy in the literature in regard to the role p38 during apoptosis has been alleviated upon discovering that p38α and β regulate apoptosis in opposite ways: p38α promotes while β inhibits apoptosis. JNK1 and JNK2 have also been regarded both as pro-apoptotic and anti-apoptotic.

Fas is shown to activate all three members of MAPK: ERK's anti-apoptotic role is clearly established but the roles of p38 and JNK continue to be unclear and controversial. The cross talk between ERK, p38 and JNK during Fas mediated cell death also remains to be elucidated. Unveiling the precise role of individual MAPK isoforms and their mechanism of action during apoptosis activated by Fas ligation will allow a better understanding of how the Fas pathway is regulated and potentially permit the development of more rational and targeted chemotherapeutic therapies that require the Fas pathway to induce apoptosis(Eischen, Kottke et al. 1997; Muller, Scaffidi et al. 1998; Poulaki, Mitsiades et al. 2001; Xia, Rosen et al. 2005). These therapies will include not only agents involved in activating the Fas pathway but will also specifically inhibit anti-apoptotic signals and potentiate pro-apoptotic signals induced by Fas.

1.7 Hypothesis and aims

On the basis of the above considerations I hypothesized that p38 and JNK differentially regulate Fas mediated apoptosis in an isoform specific manner. Since inhibition of p38 attenuated the sensitizing effect of JNK inhibition I focused on delineating the pro-apoptotic role of p38α and the anti-apoptotic role of p38β in the regulation of the initiation and the propagation of Fas-induced apoptotic signaling.

1.8 Specific objectives

I utilized Jurkat and HeLa cells to study the modulation of the apoptotic signaling events triggered by Fas activation by MAPKs. I have studied the changes in phosphorylation and

activity status of these enzymes induced by Fas. I then determined the relative importance of each MAPK branch on Fas mediated DISC assembly and regulation of the extrinsic pathway, pro-apoptotic changes at the level of the mitochondria and the intrinsic apoptotic pathway by using: 1) specific chemical inhibitors of ERK (U0126), p38 (PD169316) and JNK (SP600125), 2) si-RNA mediated translational inhibition and 3) overexpression of dominant negative (DN) variants of these kinases.

1.9 Abbreviations

MAPK: Mitogen activated protein kinases; JNK: c-jun-N terminal kinase; ERK: extracellular-signal regulated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPK kinase kinase; MKK: MAPK kinase; MEKK: MAPK kinase kinase; CD95/APO1: Fas; TNFR: Tumor necrosis factor receptor; TNF: Tumor necrosis factor; SSTR: Somatostatin receptor; SST: Somatostatin; ER: endoplasmic reticulum; FLICE: FADD like interleukin-1B converting enzyme; DD: Death domain; DED: Death effector domain; FADD: Fas associated death domain; c-FLIP: cellular FLICE-inhibitory protein;

2.0 Methods and materials

Antibodies against various proteins were obtained from commercial sources as indicated. Polyclonal antibodies against ERK, p38, p38α, JNK, caspase-9, Bid, PARP, FADD, phospho-Ser^{70} Bcl-2, Bad, site-specific phosphor-ERK1/2, phospho-Bad ($pSer^{112}$, $pSer^{136}$ and $pSer^{155}$) and monoclonal antibodies against phospho-p38 ($pThr^{180}$/p-Tyr^{182}, mAb28B10), phospho- JNK ($pThr^{183}$/pTyr185, mAbG9), anti-caspase-8 (1C12), anti-cleaved caspase-3 (5A1) and MAPK assay kits (Cell Signaling, Beverly, MA); agonistic anti-Fas (CD95/Apo-1) antibody (Fas-mAb, 2R2, monoclonal), annexin-V-FLUOS apoptosis detection kit and the complete protease inhibitor cocktail tablets (Roche Diagnostics, Montreal, QC); antibodies against c-$FLIP_{S/L}$ (H-202, monoclonal), Fas (non-agonistic, monoclonal, B10), Bax (pAbP19) and Protein A/G PLUS-Agarose (Santa-Cruz Biotechnology, Santa-Cruz, CA); anti-Bcl-2 (monoclonal, Ab1) and anti-cyt c (Ab2, polyclonal) (Oncogene, San Diego, CA); ERK and MEK1 inhibitors (U0126 and PD98959) p38 inhibitors PD169316, SB203580, SB202190 and the non functional derivative SB202474 and the JNK inhibitor SP600125 (Calbiochem, San Diego, CA); potential sensitive mitochondrial dye $DiOC_6(3)$, Sypro Ruby protein gel stain and ProQ Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR); Aminomethylcoumarin (AMC) derivatives of caspase substrates IETD, DEVD and LEHD and caspase inhibitors IETD-CHO and DEVD-CHO (BIOMOL, Plymouth Meeting, PA); Polyclonal anti-p38β antibody (Zymed, San Francisco, CA); TransIT Jurkat si-RNA transfection reagent (Mirus, Madison, WC). Double-stranded siRNAs that selectively target p38α (5'GGUCUCUGGAGGAAUUCAAtt3'-3'ttCCAGAGACCUCCUUAAGUU5'), p38β (5'-GGACUUCAGCGAAGUGUACtt3'-3ctCCUGAAGUCGCUUCACAUG5'), JNK1 (5'GGAGCUCAAGGAAUAGUAUtt3'-

3'gtCCUCGAGUUCCUUAUCAUA5') or JNK2 (5'GGGAUUGUUUGUGCUGCAUtt3'-3'ttCCCUAACAAACACGACGUA5') as well as a double stranded negative control si-RNA were purchased from Ambion (Austin, TX). I confirmed by BLAST search that these si-RNAs do not affect any known enzymes involved in the regulation of apoptosis. Plasmids encoding the Flag-tagged wild type p38α (pCMV-Flag-p38α and the inactive mutant p38α-AGF (pCMV-Flag-p38α-agf) were generously provided by Dr. R. Davis (Boston, MA).

Cell Culture: Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic, 2 mg/ml glucose, 10mM HEPES, 1mM sodium pyruvate and 2mM L-Glutamine at 37°C in a humidified atmosphere containing 5% CO_2. HeLa cells were grown in Dubeccos modified Eagle Medium.

Transfections: Exponentially growing cells were rinsed twice with PBS and suspended in 400μl serum free ice cold RPMI 1640 at 10^6 cells/ml. Eukaryotic expressing plasmid DNA (pRC-CMV-bcl-2-actA and pRC-CMV-bcl-2-cb5) was added to cells culture at a final concentration of 30 μg/ml and incubated on ice for 10 min. Then cells were electroporated using Gene Pulser™ (BIORAD) at 0.250kV and 750μF and incubated on ice for another 10 min. The transfected Jurkat T cells were selected with 800 μg/ml G418 for 3 weeks.

si-RNA transfection: cells were transfected with annealed ds-siRNA oligonucleotides as follows. Briefly, 3μl of TransIT reagent was added to 200μl of serum free medium maintained at room temperature, mixed with 60 pmol ds-siRNA and incubated for 30 min. The TransIT-siRNA complexes were then added to cells. The efficiency of si-RNA-mediated translational silencing

of each enzyme was monitored by immunoblot analysis at various times. Maximal reduction in the protein levels was observed at 48 h.

Fas mediated activation of Jurkat or HeLa cells: To determine Fas-mediated regulation of MAP kinases and incidence of apoptosis, cells were incubated with 150ng/ml of Fas-mAb for the indicated periods of time. In experiments designed to test the effect of inhibitors of p38 and JNK, cells were pre-incubated for 30 min in medium containing PD169316 or SP600125 alone or in combination prior to addition of Fas-mAb.

Detection of Apoptosis: Cells were washed and labeled with Annexin-V-FITC and propidium iodide (PI) using the Annexin-V-FLUOS staining kit according to the manufacturer's instructions. Apoptotic cells were detected by the Annexin-V-FITC labeling in the absence of PI uptake by flow cytometry. At least 20,000-gated events were enumerated for each sample and analyzed by WinList software (Verity Software House, ME).

Caspase activity measurements: AMC derivatives of DEVD, IETD and LEHD were used as fluorogenic substrates to measure the activities of caspases 3, 8 and 9 respectively. The activities were measured by monitoring the increase in fluorescence emission at 460 nm after exciting at 380 nm and quantified against a standard curve generated using 0-1000 nM AMC. To minimize cross reactivity, inhibitors of caspase-3 (DEVD-CHO) or caspase-8 (IETD-CHO) were included at a concentration of 1.5 μM when measuring the activities of caspase-8 and -3 respectively.

Immunoblot analysis: Cells were lysed in 50mM Tris-HCl buffer (pH 8.0) containing 1% NP-40, 150mM NaCl, 1mM EDTA 0.05% SDS) and protease inhibitors. Aliquots of lysates containing 40μg protein were subjected to immunoblot analysis with antibodies as indicated and detected by appropriate horseradish peroxidase-conjugated secondary antibodies and the signals captured in an Alpha Innotech Imager (San Leandro, CA).

Immunoprecipitation of Fas-associated proteins: Cells were lysed in 10mM Tris-HCl buffer (pH 7.5) containing 1% v/v Nonidet P-40, 150 mM NaCl and 0.4 mM EDTA and containing protease inhibitors as described previously with slight modifications (Daigle, Yousefi et al. 2002). Aliquots (1 mg) of lysates were pre-cleared using normal mouse IgG and then incubated with 2 μg of the non-agonistic anti-Fas antibody in presence of 80 μl of Protein A/G Sepharose beads. Immune complexes were washed and subjected to immunoblot analysis for the detection of Fas, FADD, c-FLIP and procaspase-8. In experiments aimed at detecting c-FLIP phosphorylation, c-FLIP was immunoprecipitated, electrophoresed and detected with ProQ Diamond phosphoprotein gel stain and scanned at 590 nm in a Typhoon 9200 Imager (Amersham Biosciences). The gel was then destained according to the manufacturer's protocol and detected at 610 nm after staining with Sypro Ruby protein gel stain. Alternatively (1 mg) aliquots of lysates were pre-cleared using normal mouse IgG and then incubated with 2 μg of the non-agonistic anti-Fas antibody in presence of 80 μl of Protein A/G Sepharose beads. In order to minimize the interference of the heavy and light chains of primary antibody during the IP we employed Seize Classic (A) & (G) Immunoprecipitation Kit from Pierce (Rockland, IL). Briefly cells lysates were incubated with either anti-c-$FLIP_L$ or anti-c-$FLIP_S$ (Santa Cruz) overnight after which immobilized Protein A suspension containing a cross linking agent (DSS) was added.

Immune complexes were washed and subjected to immunoblot analysis for the detection of Fas, FADD, c-FLIP and procaspase-8. In experiments aimed at detecting c-FLIP phosphorylation, c-FLIP was immunoprecipitated, electrophoresed and detected with ProQ Diamond phosphoprotein gel stain and scanned at 590 nm in a Typhoon 9200 Imager (Amersham Biosciences).

Metabolic Labeling and detection of ^{32}P labeled c-$FLIP_L$ and c-$FLIP_S$: The cells were first incubated in phosphate-free minimal essential medium for 48 h in order to deplete endogenous phosphate, after which they were incubated in fresh medium supplemented with ^{32}P labeled sodium orthophosphate (37.5μCi/well). After 24 h the cells were washed and incubated for 4h in phosphate-free medium. Cells were then lysed and protein concentration in the lysates determined. Cell lysates containing 1 mg protein aliquots in 0.5 ml lysis buffer was pre-cleared by incubation with 30 ml of Sepharose-CL 4B for 3 h at 4°C. Radioactively labeled c-$FLIP_S$ and c-$FLIP_L$ were then immunoprecipitated by incubating with anti-c-FLIP S/L antibody (10 μg) and protein using the Seize Classic (A) & (G) Immunoprecipitation Kit from Pierce (Rockland, IL). Immunecomplexes were pelleted by centrifugation for 5 min at 14, 000 x g, washed five times with the lysis buffer and released from the beads by boiling for 5 min in SDS sample buffer. The samples were resolved by SDS-PAGE, the gels dried and the radioactive bands were visualized using the Cyclone Storage Phosphor System (Perkin Elmer, Torrance, CA) following autoradiography.

Kinase activity assays: Cell extracts containing equal protein concentrations were incubated with the respective phospho-specific antibodies and immunoprecipitated using Protein A/G Sepharose

beads. The activities of the immunoprecipitated phospho-enzymes were detected by in vitro phosphorylation of Elk-1 (ERK1/2) ATF-2 (p38) and c-jun (JNK).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$): The mitochondrial potential sensitive dye $DiOC_6$ (3) (10 μM) was added to the cells 30 min prior to the conclusion of treatment with Fas-mAb. Cells were washed with PBS, excited at 360 nm and the fluorescence emission was captured at 560-nm by flow cytometry.

Subcellular fractionation: Cells were washed with PBS and lysed on ice for 10 min with the cytosol extraction buffer (4.3mM Na_2HPO_4, 1.4mM KH_2PO_4, 250mM sucrose, 70mM KCl, 137mM NaCl, 200μg/ml of digitonin and protease inhibitors) and centrifuged at 3000 x g for 5 min to obtain the cytosolic fraction. The pellet was washed three times with cytosol extraction buffer and lysed with 50mM Tris-HCl buffer (p7.5) containing 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.2% v/v Triton-X100, 0.3% NP-40, 0.5% sodium deoxycholate and protease inhibitors), incubated on ice for 15 min, and the mitochondrial fraction was recovered in the supernatant following centrifugation at 10,500 x g for 10 min at 4^0C.

Data analysis: All values had a mean ± SE, n=6.

3.0 Results

3.1 Fas-signaled apoptosis in Jurkat cells activates p38, JNK and ERK.

Fas mediated apoptosis was detectable by 4 hours and was maximal by 24 hours (Fig. 1A). p38, JNK and ERK are activated in response to Fas ligation in Jurkat cells in a time dependent manner as evidenced by the increase in phosphorylation of p38, JNK1/2 and ERK1/2 (Fig. 1B, blots 1, 3 and 5 respectively) in the absence of any change in the total level of the enzyme proteins (Fig. 1B, blots 2, 4 and 6 respectively). The increase in phosphorylation correlated with the increase in their *in vitro* kinase activities as demonstrated by the ability of p38 to phosphorylate ATF-2 (Fig. 1C, blot 1), of JNK to phosphorylate c-jun (Fig. 1C, blot 2) and of ERK to phosphorylate Elk-1 (Fig. 1C, blot 3) following immunoprecipitation with the respective phospho-specific antibodies.

In order to assess the modulatory influence of MAPKs on Fas-signaled apoptosis I evaluated the effect of synthetic inhibitors of ERK (U0126), p38 (PD169316) and JNK (SP600125) on Fas-mediated cell death in Jurkat cells. SP600125 and PD169316 increased the apoptotic responsiveness to Fas-ligation by 4 and 2.5-fold respectively (45.3 ± 0.7 and 26.9 ± 0.5 % compared to 11.4 ± 0.6 % observed in their absence, Fig. 1D, left panel). By contrast, in presence of ERK inhibitor U0126, Fas-mediated cell death was only slightly higher than that seen in its absence (17.8 ± 0.6% vs. 11.4 ± 0.6 %, Fig. 1D, left panel). These inhibitors did not, at the concentration tested, trigger apoptosis in the absence of Fas-mAb treatment (Fig. 1D, right panel). I next compared the effects of U0126, PD169316 and SP600125 in various combinations

in order to determine if the three arms of the MAPK signaling pathways exert synergistic or antagonistic effects on Fas-mediated apoptosis (Fig. 1E). The potentiating effect of SP600125 was attenuated by PD169316 (lane 5) with the level of Fas-mediated apoptosis reduced to 24.0 ± 1.0 %, a value similar to that seen in presence of the latter inhibitor alone (compare lanes 1 and 5). U0126 did not influence the potentiating effect of PD169316 (29.2 ± 1.5% vs. 26.9 ± 0.5 %, Fig. 1E, compare lanes 1 and 2) or of SP600125 (45.3± 0.7% vs. 48.3 ± 1.5%, compare lanes 3 and 4). Moreover, the attenuation of the sensitizing effect of SP600125 by PD169316 was not affected by U0126 (lane 6). The observation that p38 is required for the sensitization of apoptosis by JNK inhibition was confirmed by the ability of two other inhibitors of p38 (SB202190 and SB203580) to abrogate the sensitizing effect of SP600125 (36 ± 2% and 34 ± 1% apoptotic cells respectively, Fig. 1E, lanes 7 and 8) and by the lack of effect of the non-functional derivative SB202474 (lane 9). These results indicated that Fas-signaled apoptosis is potentiated by the inhibition of JNK in the presence, but not the absence, of p38 activation. This was not influenced by ERK.

The specificity of the inhibitors was confirmed by their ability for inhibiting *in vitro* kinase activities in lysates of cells incubated with Fas-mAb in the absence or presence of the inhibitors. PD169316 inhibited phosphorylation of ATF-2 by p38 (Fig. 1F, blot 1, lane 2 vs. 3), SP600125 selectively inhibited JNK-mediated phosphorylation of c-jun (Fig. 1F, blot 2, lane 2 vs. 3) and U0126 inhibited the phosphorylation of Elk-1 by ERK (Fig. 1F, blot 3, lane 2 vs. 3). Given the observed interactions between p38 and JNK, I wanted to verify if inhibition of p38 did not alter the activity of JNK. Therefore insuring that the effect mediated by p38 inhibition during Fas mediated sensitization during JNK inhibition is specific. PD169316 inhibited phosphorylation of

ATF-2 by p38 whereas SP600125 selectively inhibited JNK-mediated phosphorylation of c-jun (Fig.1G, blots 1 and 2 - compare lanes 3 and 4 vs. lane 2). Both kinases were inhibited only in cells treated with the two inhibitors together as shown by the lack of phosphorylation of ATF-2 and c-jun (lane 5). In lysates of cells incubated in the absence of Fas-mAb (lane 1) of the inhibitors neither p38 nor JNK activities could be detected in measurable quantity under identical conditions.

3.2 p38α and p38β differentially influence Fas mediated cell death in Jurkat cells.

The activation and presence of JNK1/2 isoforms (p46/p54) and ERK1/2 (p44/p42) can be observed in Figure 1A based on the different sizes of these isoforms. However this is not the case for the p38 isoforms (Fig. 1A, blots 1 and 2). All five isoforms of p38 display the same electrophoretic mobility on SDS-PAGE (38kDa). In order to study PD169316-sensitive isoforms of p38 (p38 α and β) that are ubiquitously expressed, total p38 was immunoprecipitated with an anti-p38 antibody that recognized both p38α and 38β and subsequent immunoblot analysis with antibodies specific for the individual isoforms confirmed their presence in Jurkat cells (Fig. 2A, blots 2 and 3). The levels of either isoform or total p38 were not altered in response to Fas-activation (Fig. 2A, blots 1, 2 and 3: lane 2). The increased phosphorylation of p38α and p38β in Fas-ligated cells was confirmed by a similar approach, but by using isoform-specific anti-p38 antibodies to pull down the kinases and immunoblotting with phospho-specific anti-p38 antibody (Fig. 2B, blots 1 and 2: lane 1 vs 2).

I then sought to determine the effect of inhibiting individual isoforms of p38 using two different techniques. The first is si-RNA mediated translational silencing and the second is the transient over expression of these kinases and their dominant negative (DN) variants in Jurkat cells. I generated cells lacking p38α, β and JNK1/2 using the si-RNA strategy and labeled these cells as si-p38α, si-p38β and si-JNK1/2 cells respectively. I also generated cells over-expressing p38α, p38β and JNK1/2 and labeled them as Flag-p38α, Flag-p38β and Flag-JNK1/2 respectfully. Cells expressing the dominant negative variants of p38α and JNK1/2 were also produced and labeled Flag-p38α-AGF and Flag-JNK1/2-AGF respectively.

In si-p38α cells the expression of p38α was completely inhibited without affecting that of p38β (Fig. 2C, compare lane 2 in blots 1 and 2). In si-p38β cells there was a significant inhibition of the expression of p38β (Fig. 2C, lane 4) but not p38α (lane 2). As shown in these blots, transfection of a control si-RNA (si-C) did not affect the expression of both isoforms of p38 (Fig. 2C, blot 1 and 2, lane 3). In parallel, I also generated cells lacking JNK isoforms 1 and 2 (si-JNK1/2) and confirmed the suppression of their expression (Fig. 1D). The dominant negative plasmid coding for Flag-tagged p38β was not available and could not be tested. The expression of Flag tagged proteins was confirmed by immunoblot analysis using anti-p38α antibody (Fig. 2E, left panel) and anti-Flag antibody (Fig. 2E, left panel).

Si-p38α, si-p38β and si-JNK1/2 cells were then treated with Fas to validate the data I obtained using the chemical inhibitors against MAPKs. Apoptosis was substantially inhibited in si-p38α cells and enhanced in si-p38β and si-JNK1/2 cells (5.4 ± 0.4%, 41.3 ± 1% and 46 ± 2.8% compared to 12 ± 1.2 in si-C cells, Fig. 2F).

The effects of PD169316 and SP600125 individually during Fas treatment in si-C, si-p38α, si-p38β and si-JNK1/2 cells were then determined (Fig. 2G). Fas treated cells in the presence of PD169316 induced more than a five fold increase in sensitization in si-p38α cells compared to the two fold increase noted in si-C cells (compare lanes 2 and 3 vs. 6 and 7). This was in contrast to the 50% decrease in apoptotic levels induced by PD169316 treatment observed in si-p38β cells (lane 10 vs. 11) and si-JNK1/2 cells (lane 14 vs. 15). The latter observation concerning JNK1/2 cells confirms the data we obtained by concomitant use of SP600125 and PD169316 (Fig 1E, lane 5). Sensitization mediated by SP600125 was blunted in si-p38α cells only (lane 8 vs., 4, 12 and 16). Fas-mediated apoptosis was higher in presence of SP600125 than in its absence in si-C cells (48.7 ± 0.8% vs. 12 ± 1.2%, lane 2 vs. 4) and si-p38α cells (18.5 ± 0.8% vs. 5.4 ± 0.4%, lane 6 vs. 8), but not si-p38β cells (45.3 ± 1.1% vs. 41.3 ±1%, lane 10 vs. 12). PD169316 inhibited Fas mediated apoptosis in si-JNK1/2 cells

Jurkat cells over-expressing wild type p38α, β, JNK1/2 and the dominant negative variants of p38α and JNK1/2 were then treated with Fas to confirm the data obtained using either chemical inhibitors against MAPK or translational silencing of these enzymes. Cells over-expressing wild type p38α (Flag-p38-α) showed increased levels of apoptosis while over-expression of the DN variant of p38α (Flag-p38-α-AGF) inhibited Fas mediated apoptosis (Fig. 2H, compare lanes 3 and 4). Cells over-expressing wild type JNK1/2 (Flag-JNK1/2) inhibited Fas mediated cells death (compare lanes 2 and 6) while cells expressing the DN JNK1/2 (Flag-JNK1/2-AGF) potentiated Fas mediated cell death (compare lane 2 with lanes 6 and 7). Cells over-expressing wild type p38β blocked Fas mediated cell death (compare lanes 2 and 5 and then compare Fig.

2D, lane 10 with Fig. 2H lane 5). In absence of Fas ligation, transient over expression of the wild type p38α, p38β and JNK1/2 and the DN variants of p38α and JNK1/2 did not induce apoptosis (Fig 2H, lanes 8-12)

3.3 Inhibition of p38 and JNK, but not ERK, alters Fas mediated caspase-8 activation.

I observed that the induction of MAPK in Fas-activated cells preceded the activation of caspase-8 since the presence of cleaved caspase-8 fragments p44/43 and p16 was detectable only beyond 2 h in Fas-activated Jurkat cells (Fig. 3A). The in vitro caspase-8 activity (measured using IETD-AMC as the substrate) was higher in extracts of Fas-ligated cells in presence of SP600125 and PD169316 (Fig. 3B, 0.530 ± 0.007 (lane 5) and 0.397 ± 0.020 nmol/μg/min (lane 4) respectively) than in their absence (0.183 ± 0.017 nmol/μg/min (lane 2)). By contrast, the ERK inhibitor U0126 did not significantly alter the extent of Fas-mediated activation of caspase-8 (0.201 ± 0.013 (lane 3) nmol/μg/min vs 0.183 ± 0.017 nmol/μg/min (lane 2)). This confirmed our initial observation that ERK inhibition did not significantly impact Fas mediated cell death in Jurkat cell. I therefore focused my attention on the modulatory effects of p38 and JNK during Fas signaled apoptosis. Concomitant treatment with PD169316 and SP600125 during Fas signaled apoptosis did not alter increased caspase-8 activity induced by treatment with SP600125 alone (lane 6); this indicates that SP600125 mediated sensitization is p38 dependent distal to caspase-8 activation.

The observed differences in the activities of these caspases correlated with the increase in the amounts of cleaved fragments of procaspase-8 in cells treated with these inhibitors individually

or together (Fig 3C, blot 1) and the presence of tBid, the cleavage product of the caspase-8 substrate Bid, as assessed by immunoblot analysis (Fig 3C, blot 2). Fas-induced generation of caspase-8 was blunted in si-p38α cells (Fig 3D, lane 2) but was enhanced in si-JNK1/2 (Fig. 3E, lane 3) and si-p38β cells (Fig. 3D, lane 4)

In order to confirm the role of individual p38 isoforms I compared the effects of PD169316 and/or SP600125 in si-C, si-p38α, si-p38β and si-JNK1/2 cells. As previously observed caspase-8 activation is completely inhibited in si-p38α cells whereas it is increased in p38β and JNK1/2 cells compared to si-C cells (Fig. 3F, compare lane 2 to lane 6, 10 and 14). PD169316 potentiated Fas mediated activation of caspase-8 in si-C (lane 3) and si-p38α (lane 7) cells, inhibited it in si-p38β (lane 11) and had no effect in si-JNK1/2 (lane 15) cells. SP600125 did potentiate Fas mediated activation of caspase-8 in si-C and p38α cells but not in si-p38β and si-JNK1/2 cells.

3.4 Effect of p38α, p38β, JNK1/2 and ERK1/2 on DISC assembly.

Since caspase-8 activation occurs within the DISC complex, I investigated the effect of inhibiting p38, JNK and ERK on DISC assembly. In order to elucidate the changes in DISC composition during Fas treatment, I isolated DISC components associated with Fas by immunoprecipitating Fas using a non-agonistic anti-Fas antibody and identified bound DISC components (pro-caspase-8, Fas, FADD and c-FLIP$_S$) by immunoblot analysis (Fig. 4A). Total Fas levels remained unchanged during Fas treatment while a time dependent increase in FADD levels with a concomitant decrease in the levels of c-FLIP$_S$ was observed. Pro-caspase-8 levels

within the DISC complex increase with time for up to three hours, however at 4 hours there is a substantial decrease suggesting activation and subsequent release of caspase-8 from within the DISC into the cytoplasm.

Inhibition of ERK, with U0126, did not alter DISC composition in Jurkat cells (Fig. 4B), thus confirming our observation that ERK did not play a significant role in modulating the initiation of Fas mediated apoptotic signaling in Jurkat cells. The effect of chemical MAPK inhibitors on Fas expression and its association with c-FLIP$_S$, c-FLIP$_L$, FADD and procaspase-8 was then investigated. Inhibition of JNK (Fig. 4C, left panel, lane 3) and p38 (Fig. 4C, left panel, lane 2) induced a loss of Fas-associated c-FLIP$_S$ but not c-FLIP$_L$ and a concomitant increase in Fas associated FADD. An increase in the amount of procaspase-8 present in the immune complex was evident under these conditions. PD169316 did not effect SP600125 induced alteration in DISC composition (Fig. 4C, lane 4). This is in agreement with the observation that the increase in caspase-8 activation in Fas-activated cells treated with SP600125 was not affected by the presence of PD169316 and by the detection of comparable amounts of cleaved fragments procaspase-8 by immunoblot analysis.

Fas-ligation induced the expected increase in its association with FADD and procaspase-8 with a concomitant decrease in Fas-associated c-FLIP$_S$ after 1 h treatment with Fas-mAb (Fig. 4C, right panel, lane 5) a time point at which autoproteolytic cleavage of procaspase-8 was undetectable. These changes were not due to altered levels of immunoprecipitated Fas (Fig. 4C, bottom panel), or of total FADD or c-FLIP$_S$ in whole cell lysates (Fig. 4C, bottom panel). The reduction in c-FLIP$_S$ and the increase in FADD and procaspase-8 in DISC induced by the PD169316 (Fig. 4C,

right panel, lane 6) and SP600125 (lane 7) were more pronounced in Fas-ligated cells than in control cells (left panel). DISC bound c-$FLIP_L$ levels remained unchanged in absence (Fig 4C, left panel) or presence (Fig. 4C, right panel) of Fas ligation in Jurkat cells. Total levels of c-$FLIP_L$ also were unaffected in these two conditions (Fig 4C, bottom left and right panels).

Fas expression and DISC complex in Jurkat cells transfected with p38α, p38β, JNK1/2 or the negative control si-RNAs was then compared. A striking difference in the DISC composition was observed in si-p38α and si-p38β cells: a marked reduction in the presence of c-$FLIP_S$ and a concomitant increase in FADD and procaspase-8 was seen in si-p38β cells whereas DISC composition remained unchanged in si-p38α cells and si-C cells (Fig. 4D, top panel, compare lane 3 with lanes 2 and 1). Increased presence of FADD and procaspase-8 was also observed in si-JNK1/2 cells (lane 4). These changes were not due to altered levels of immunoprecipitated Fas (Fig. 4D, top panel), or of total FADD, procaspase-8 and c-$FLIP_S$ in whole cell lysates (fig 4D, bottom panel). DISC bound c-$FLIP_L$ levels remained unchanged in all si-RNA transfected cells (Fig 4D). Total levels of c-$FLIP_L$ were also unaffected (Fig 4D, bottom panel).

Given the striking difference in the presence of c-$FLIP_S$ in the DISC in cells lacking p38α and p38β, I immunoprecipitated c-$FLIP_S$, electrophoresed and determined its phosphorylation status by pro-Q diamond staining. The pro-Q diamond stain is a new fluorescent method of detecting phosphorylation within in acrylamide gels and does not require western blotting or use of phospho-specific antibodies. The stain detects phosphate groups attached to tyrosine, serine or threonine residues. Increased phosphorylation of c-$FLIP_S$ was seen in si-p38α and si-C cells but not in si-p38β or si-JNK1/2 cells (Fig. 4E, top panel, compare lanes 1 and 2). By contrast, there

was almost no phosphorylation of c-FLIP$_S$ in si-p38β or si-JNK1/2 cells (lanes 3 and 4). The levels of total c-FLIP$_S$ in these cells were comparable as demonstrated by Sypro Ruby protein staining (Fig. 4E, bottom panel).

These findings were further confirmed by monitoring the phosphorylation of c-FLIP in cells metabolically labeled with radioactive ^{32}P. The increase in phosphorylation of c-FLIP$_S$ seen in cells transfected with si-p38α was higher than that in cells expressing Flag-p38α-AGF (Fig 4F, compare lanes 3 and 4). Over-expression of wild type p38α resulted in a suppression of c-FLIP$_S$ phosphorylation (lane 5) to the level seen in the native and in control si-RNA transfected Jurkat cells (lanes 1 and 2). By contrast, such manipulation of p38α activity did not affect the phosphorylation status of c-FLIP$_L$.

3.5 Modulation of Fas mediated activation of the mitochondrial apoptotic pathway by p38α, p38β and JNK1/2.

I next evaluated the effect of p38α, p38β and JNK on mitochondrial proapoptotic activity. I focused my attention on the mitochondria based on the data I obtained on the differential modulation of p38α, p38β and JNK1/2 on caspase-8 activation and Bid cleavage. I first studied accessed the effect of p38 and JNK inhibitors on the mitochondrial membrane potential $\Delta\psi_m$. A reduction in $\Delta\psi_m$ indicates increased mitochondrial membrane permeability and subsequent release of pro-apoptotic factors from within the mitochondria into the cytoplasm. A significant increase in the number of cells with reduced $\Delta\psi_m$ was observed following treatment with Fas-mAb in the presence of SP600125 than in its absence (Fig. 5A, 58 ± 4 vs. 33 ± 2 %). PD169316

inhibited SP600125-induced increase in the number of cells with decreased $\Delta\psi_m$, (28 ± 5 %) but not the effect of Fas-mAb itself (compare lanes 2, 3, 4 and 5). In the absence of Fas-mAb treatment, these inhibitors did not decrease $\Delta\psi_m$ (Fig. 5A, right panel).

The potentiation of Fas-mediated reduction in $\Delta\psi_m$, and the suppression of the sensitizing effect of SP600125 by PD169316 were confirmed with two other pharmacological inhibitors of p38 - SB202190 and SB203580 - and by the lack of effect of the nonfunctional derivative SB202474 (Fig. 5B).

I confirmed the data obtained using chemical inhibitors against p38 and JNK by employing si-RNA mediated translational silencing of these two kinases. The reduction in $\Delta\Psi m$ in response to Fas-activation was also much lower in si-p38α cells (11.3 ± 0.6%) but not in si-p38β cells (36 ± 0.9%) compared to the value of 33 ± 2% in si-C cells (Fig. 5C). Interestingly, the reduction in $\Delta\Psi m$ was much greater in si-JNK1/2 cells (74 ± 1.4%). In order to confirm the data I observed when inhibiting p38 and JNK simultaneously using chemical inhibitors, I treated si-C, si-p38α, si-p38β and si-JNK1/2 cells with either PD169316 or SP600125 and measured $\Delta\psi_m$. As previously observed loss of $\Delta\psi_m$ is completely inhibited in si-p38α cells whereas it is unchanged and increased in si-p38β and si-JNK1/2 cells respectively compared to si-C cells (Fig. 5D, compare lane 2 to lane 6, 10 and 14). PD169316 increased Fas mediated loss of $\Delta\psi_m$ in si-p38α but did not affect Fas mediated loss of $\Delta\psi_m$ si-p38β cells.

I then treated Jurkat cells over-expressing wild type p38α, β, JNK1/2 and the dominant negative variants of p38α and JNK1/2 with Fas to confirm the data I obtained using either chemical

inhibitors against MAPK or translational silencing of these enzymes. Cells over-expressing wild type p38α (Flag-p38-α) showed increased loss of $\Delta\psi_m$ while over-expression of the DN variant of p38α (Flag-p38-α-AGF) inhibited Fas mediated reduction of $\Delta\psi_m$ (Fig. 5E compare lanes 3 and 4). Cells over-expressing wild type p38β (Flag-p38β) and JNK1/2 (Flag-JNK1/2) did not alter Fas mediated reduction of $\Delta\psi_m$ (compare lane 2 with lanes 5 and 6 respectively) while cells expressing the DN JNK1/2 (Flag-JNK1/2-AGF) potentiated Fas mediated loss of $\Delta\psi_m$ (compare lanes 2 and 7. Cells over-expressing DN p38β were not available in time for completing this analysis. In absence of Fas ligation (Fig 5E, lanes 8-12), over-expression of the wild type or DN p38 and JNK did not induce loss of $\Delta\psi_m$.

My findings indicate that p38α promoted the activation of DISC. However, its specific influence at the level of the mitochondria remained unknown. To elucidate p38α's specific actions at the level of the mitochondria I compared the effect of Fas and/or mitochondrial toxin staurosporine (STS) in si-C, si-p38α, si-p38β and si-JNK1/2 cells. STS is known to induce apoptosis in various cell lines by directly inducing mitochondrial proapoptotic activity. I treated the si-RNA treated cells to Fas and STS separately and concomitantly and accessed apoptosis induction. STS potentiates Fas mediated apoptosis in si-C cells (Fig. 5F, compare lane 2 and 3 vs. lane 4). STS induced apoptosis remains unaffected in si-p38α cells (compare lane 3 and 7); indicating that Fas mediated loss of $\Delta\psi_m$ during p38α inhibition is dependent on the modulatory effect of p38α on DISC composition and not a distinct concomitant effect at the level of the mitochondria. Fas and STS induce apoptosis in si-p38β and si-JNK1/2 cells.

3.6 Effect of p38 and JNK inhibition on Fas mediated activation of caspases -9 and -3.

Fas-mediated activation of caspase-9 was lower in cells incubated in the presence of PD169316 than in its absence (Fig 6A, top panel, lanes 2 and 3: 0.30 ± 0.003 vs. 0.17 ± 0.04 nmol/μg/min), but was higher in cells treated with SP600125 (0.56 ± 0.006 nmol/μg/min, lane 4). In cells treated with both inhibitors, caspase-9 activity decreased to the level comparable to that seen with PD169316 alone (0.17 ± 0.04 vs. 0.24 ± 0.04 nmol/μg/min, compare lanes 3 and 5). Likewise caspase-3 (DEVD-ase), a substrate of caspase-9, activity was greater in presence of SP600125 than PD169316 (Fig. 6A, bottom panel, 0.53 ± 0.007 and 0.40 ± 0.02 respectively vs. 0.19 ± 0.005 nmol/μg/min in their absence). Fas-induced formation of cleaved fragments of caspases 9 and 3 was maximal in cells treated with SP600125 in the absence, but not presence of PD169316 (Fig. 6B blots 1 and 3) and was reflected by the parallel changes in the cytosolic accumulation of cytochrome c (blot 2) and caspase-3 substrate PARP (blot 4) respectively. As shown in these blots, the potentiating effect of SP600125 on the activation of caspases-9 and -3 in Fas-ligated cells was abrogated by PD169316 (compare lanes 4 and 5) to the level seen in the presence of the latter alone (lane 3).

Fas-induced generation of caspases-9 and -3 was blunted in si-p38α cells (Fig. 6C, blot 1 and 2) but was enhanced in si-JNK1/2 cells (Fig. 6D, blots 1 and 2). In si-p38β cells, on the other hand, enhanced formation of caspases-9 and -3 was observed (Fig. 6C). The differential effects of p38α and p38β on caspase-9 activation correlated with the finding that abrogation of p38α expression inhibited cytochrome c release from the mitochondria while that of p38β was without effect (Fig. 6C, blot 3) while it was higher in si-JNK1/2 cells as expected (Fig 6D, blot 3).

Fas-activation led to the activation of caspase-9 (Fig. 6E, top panel: from 0.03 ± 0.001 to 0.34 ± 0.03 nmol/μg/min) and caspase-3 (Fig. 6E, bottom panel: 0.04 ± 0.002 to 0.20 ± 0.02 nmol/μg/min) in si-C cells. By contrast, in si-p38β cells, there was a significantly higher increase in the activity caspase-3 (0.55 ± 0.03 nmol/μg/min) but not caspase-9 (0.25 ± 0.03 nmol/μg/min) whereas there was no significant Fas-mediated activation of caspases-9 and -3 in si-p38α cells. The effect of PD169316 on Fas-mediated activation of caspase-3 revealed a similar pattern, being higher in si-C and p38α cells and lower in si-p38β cells compared to the values observed following Fas-activation in the absence of this inhibitor. By contrast, caspase-9 was activated in si-C and si-p38β, but not in si-p38α cells. Of particular interest was the finding that PD169316 completely abrogated Fas-mediated activation of caspase-9 in si-p38β cells (from 0.25 ± 0.04 to 0.06 ± 0.01 nmol/μg/min) but only partially in si-C cells (0.21 ± 0.01 from 0.34 ± 0.04 nmol/μg/min). As expected, SP600125 did not exert any effect in si-JNK cells. Moreover, PD169316 inhibited the sensitizing effect of SP600125 on Fas-mediated activation of caspases-9 and -3 in si-p38α cells and si-JNK1/2 cells but not in si-p38β cells.

3.7 Effect of MAPK inhibition on pro- and anti-apoptotic Bcl-2 family members during Fas mediated apoptosis.

The differential regulatory effects of p38 and JNK on mitochondrial function in apoptosis suggested that they might influence the mitochondrial sequestration and/or activation of proapoptotic proteins of the Bcl-2 family such as Bax, Bak, tBid and Bad. Immunoblot analysis of the mitochondrial and cytosolic fractions revealed that PD169316, but not SP600125, prevented the mitochondrial localization of Bax. Most striking was the effect of PD169316 to

inhibit the translocation of Bax to the mitochondria both in the absence and presence of SP600125 (Fig. 7A - blots 1 and 2, compare lanes 2-5). The presence of tBid in the mitochondria was seen in Fas-ligated cells in the absence and presence of SP600125, but not PD169316 (blot 5). However, the mitochondrial presence of tBid in Fas-ligated cells was unaffected in cells treated with both inhibitors. The presence of Bak in mitochondrial fraction was unaffected under these experimental conditions (blot 3 and 4). These inhibitors differentially influenced the phosphorylation status of Bad. In Fas-activated cells, there was a loss of phosphorylation at Ser^{112} and Ser^{155}, but not at Ser^{136}. Whereas PD169316 reversed the loss of Bad phosphorylation at Ser^{112} and Ser^{155}, SP600125 treatment led to the loss of Ser^{136} phosphorylation (blots 6, 7 and 8, compare lanes 2,3 and 4). These changes in Bad phosphorylation occurred in the absence of any change in the total levels of Bad (blot 9). Ser^{70} phosphorylation of mitochondrial Bcl-2 was inhibited in the presence of PD169316, but not SP600125 (blot 10, compare lanes 2, 3 and 4). The mitochondrial presence of Bcl-2 was not altered by Fas-ligation in the absence or presence of the inhibitors of p38 and JNK (blot 11). The efficacy of subcellular fractionation was confirmed by the detection of the mitochondrial marker TOM-20 exclusively in mitochondrial fractions (blot 12). The cytosolic fraction did not show any presence of TOM 20 (data not shown)

Mitochondrial translocation of Bax did not occur in response to Fas-activation in both si-p38α and si-p38β cells (Fig. 7B - blots 1 and 2, compare lanes 3 and 4 to lane 2). On the other hand, the mitochondrial localization of Bax in si-JNK1/2 cells was higher than in si-C cells (blots 1 and 2, compare lanes 2 and 5). Cleavage of Bid into tBid (blot 3) and translocation of tBid to the mitochondria occurred in si-C, si-p38β and si-JNK1/2 cells, but not in si-p38α cells (blot 4). Fas-

induced loss of phosphorylation of Bad at Ser^{112} and Ser^{155} was prevented in both si-p38α and si-p38β cells (blots 5 and 7, compare lanes 2, 3 and 4). Bad phosphorylation at Ser^{136} was absent uniquely in si-JNK1/2 cells (blot 6, lane 5). Total Bad levels were comparable in these cells (blot 8). There was no change in mitochondrial Bcl-2 in these cells (blot 10). However, Ser^{70} phosphorylation of Bcl-2 was abrogated in si-p38α, but not si-p38β or si-JNK1/2 cells (blot 9).

3.8 ER but not mitochondrial localized Bcl-2 blunts sensitization to Fas mediated apoptosis during MAPK inhibition in Jurkat and HeLa cells.

The results in Fig. 7 suggested that Fas mediated activation of MAPK regulates the function of Bcl-2 proteins. I therefore investigated the effect of targeted overexpression of Bcl-2 at the mitochondria and the ER and studied the effect of this over-expression on Fas mediated apoptosis during p38 and JNK inhibition. I prepared stable transfectants of Jurkat and HeLa cells over-expressing Bcl-2 at level of the mitochondria (Bcl-2-actA) and endoplasmic reticulum (Bcl-2-cb5). Over-expression of Bcl-2 at the level of the mitochondria (Fig. 8A and D, middle panels) decreases Fas induced levels of apoptosis in both Jurkat and HeLa treated with chemical inhibitors. However, Bcl-2 over-expression at the level of the ER (Fig. 8A and D, right panels) completely abrogates Fas induced apoptosis and its sensitization by the inhibition of p38 and JNK in both cell lines.

Immunoblot analysis reveals that Bcl-2 is over-expressed in both Jurkat (Fig. 8B) and HeLa cells (Fig. 8E). These transfected cells over-express fusion proteins which in the case of Bcl-2-actA are about the same molecular weight as Bcl-2 whereas the fusion of Bcl-2 with the ER anchoring

domain of cytochrome B5 is somewhat larger than endogenous Bcl-2 (lane 4). Bcl-2 does not affect Fas mediated activation of caspase-8 (Fig. 8C, top panel) but does block caspase-3 activation (Fig. 8C, bottom panels) in Jurkat cells.

3.9 ERK1/2 activation inhibits induction of anti-apoptotic p38α.

My data have shown that HeLa cells (Fig 8D, lane 2), unlike Jurkat cells are insensitive to Fas-induced apoptosis. However, inhibition of ERK sensitizes HeLa cells (Fig 9F, compare lanes 2 and 3) to a greater extent compared to Jurkat cells during Fas signaled apoptosis. I suspected that differential MAPK activation profiles between both cells lines might explain the differences in Fas responsiveness in these two different cell lines. I treated HeLa cells with Fas and evaluated the activation of MAPK. Fas treatment induces a robust phosphorylation/activation of both ERK (Fig. 9A, blot 1) and p38 (Fig. 9A, blot 3) by 30 minutes and was sustained for up to 24 hours. Total levels of these enzymes were unaffected by Fas treatment (blots 2 and 4). Fas treatment also induces the activation of JNK up to 24 hours into treatment (Fig. 9D). Fas treatment induced the activation of p38β (Fig. 9B, blot 6) but not p38α (blot 5). Total amounts of each isoform (p38α and p38β) were unaffected during Fas treatment (blots 7 and 8). This is in contrast to Jurkat cells where both p38 α and β are both activated and were insensitive to the effect of ERK inhibition during Fas signaling (Figure 1). Simultaneous treatment of HeLa cells with Fas and ERK inhibitor PD98059 revealed that p38α was activated upon inhibition of ERK (Fig. 9C, blot 9). ERK inhibition also induces an activation p38β even in the absence of Fas treatment (blot 10).

As observed in Jurkat cells sensitization of Fas mediated cell death due to inhibition of JNK requires active p38 (Fig. 9E, compare lanes 4 and 5). Concomitant treatment of HeLa cells with Fas-mAb and ERK inhibitors (Fig. 9F, U0126 (lane 6) or PD98059 (lane 3)) or p38 inhibitor PD169316 (lane 4) sensitizes these cells to Fas mediated apoptosis. Upon concomitant inhibition of p38 and ERK, I noticed that PD98059 (lane 5) or U0126 (lane 7) mediated sensitization was abrogated by the presence of PD169316.

4.0 Discussion

In this study I demonstrated that Fas-mediated apoptosis in Jurkat cells and its sensitization by the inhibition of JNK are dependent on p38α-regulated inhibition of c-FLIP$_S$ phosphorylation and its exclusion from the DISC: in cells lacking p38α, increased phosphorylation and presence of c-FLIP$_S$ in DISC accounted for diminished caspase-8 activation and apoptotic responsiveness (Tourian, Zhao et al. 2004). By contrast, neither phosphorylation status of c-FLIP$_L$ nor its presence in DISC was influenced by p38α. Inhibition of Fas-mediated caspase-8 activation in p38α-lacking cells correlated with the expected inhibition of tBid formation and its mitochondrial presence. In contrast to the selective effect of p38α on mitochondrial targeting of tBid, mitochondrial presence of Bax and the loss of Bad phosphorylation at Ser^{112}/Ser^{155} were regulated by both p38α and p38β and were abrogated in cells lacking either of these p38 isoforms. Enhanced mitochondrial proapoptotic activity in response to the inhibition of JNK1/2 correlated with the loss of Bad (Ser^{136}) phosphorylation only when there was concomitant, p38α/β-dependent decrease in Ser^{112}/Ser^{155} phosphorylation. Collectively, these findings demonstrate that p38α facilitates Fas-mediated activation of caspase-8 by preventing or inhibiting the phosphorylation and presence of c-FLIP$_S$, but not c-FLIP$_L$, in DISC. Downstream of caspase-8, p38α-dependent targeting of tBid, and p38α/β-dependent sequestration of Bax in the mitochondria and inhibition of phosphorylation of Bad on Ser^{112}/Ser^{155} promoted events leading to caspase-9 activation. My findings also demonstrate that ER resident Bcl-2, but not mitochondrial Bcl-2, is able to greatly attenuate the sensitizing effect of p38 and JNK inhibition during Fas mediated cell death distal to caspase-8 in both Jurkat and HeLa cells (Tourian L. 2003). However, in the absence of MAPK inhibition, HeLa cells but not Jurkat cells, are

insensitive to Fas mediated apoptosis. My results indicate that Fas insensitivity in HeLa cells results, in part, from Fas mediated activation of ERK1/2. In HeLa cells (unlike in Jurkat cells) inhibition of ERK greatly sensitizes them to death receptor mediated cell death. I have shown here that resistance of HeLa cells to Fas signaling is partially due to ERK mediated inhibition of Fas-signaled activation of p38α. Inhibition of ERK1/2 activates p38α and induces apoptosis in Fas treated HeLa cells.

Caspase-8 is generated by the autoproteolytic cleavage of procaspase-8 within the DISC in presence of ligated Fas and the adapter protein FADD (Kischkel, Hellbardt et al. 1995; Medema, Scaffidi et al. 1997). c-FLIP binds to FADD through its DED domains and blocks the recruitment and/or the autoproteolysis of procaspase-8 (Perlman, Pagliari et al. 1999; Krueger, Schmitz et al. 2001). While such interaction appears to be regulated, in part, by multi-site phosphorylation within the DED, the mechanisms and mediators governing phosphorylation is not well established (Yang, Xiao et al. 2003). Here I have demonstrated that the phosphorylation and DISC localization of c-FLIP$_S$, but not c-FLIP$_L$ is attenuated by p38α, an effect that was enhanced by ectopically introduced Flag-tagged wild type p38α and inhibited by the depletion of p38α by si-RNA-mediated translational silencing and by the dominant negative effect of Flag-tagged inactive mutant, p38α-AGF. This, to my knowledge, constitutes the first evidence demonstrating the regulation of c-FLIP$_S$ activity by post-translational modification through p38α-dependent phosphorylation. Future studies should help determine whether p38α affects c-FLIP$_S$ phosphorylation by inhibiting kinase(s) and/or by activating phosphatase(s).

A potential candidate kinase is the calcium/calmodulin-dependent protein kinase II, which phosphorylates c-FLIP$_S$ to promote its presence in Fas-DISC and to inhibit Fas-induced apoptosis in glioma cells (Yang, Xiao et al. 2003). The selective increase in the presence of phosphorylated c-FLIP$_S$ in DISC leading to the inhibition of Fas-mediated apoptotic signaling contradicts a previously reported finding that PKC-dependent phosphorylation of both c-FLIP$_L$ and c-FLIP$_S$ is induced by bile acids in hepatocytes and promote their presence in TRAIL-DISC and enhance TRAIL-mediated apoptosis (Higuchi, Grambihler et al. 2004). Future studies aimed at identification of the sites of phosphorylation sensitive to different kinases and in particular the sites targeted in a p38α-dependent manner will be necessary to further examine the importance of site-specific phosphorylation of DED domain of c-FLIP$_{S/L}$ in the regulation of DISC activation by different death-receptor ligands. The possibility that p38α may regulate the phosphorylation status of c-FLIP$_S$ through putative phosphatase(s) cannot be excluded and remains to be explored.

Fas-mediated activation of caspase-8 was potentiated by SP600125 in si-C and si-p38β, but not si-p38α cells. However, enhanced activation of caspase-9 in presence of SP600125 was seen in Fas-activated si-C cells but not si-p38α and si-p38β cells. Moreover, PD169316 blunted Fas-mediated caspase-9 activation in si-JNK1/2 cells. Collectively these findings suggest that p38α promotes the direct caspase cascade characteristic of extrinsic or type I apoptotic signaling whereas JNK1/2 selectively inhibits type II signaling. The greater reduction in $\Delta\Psi m$, cytochrome c release and caspase-9 activation due to JNK inhibition correlated with the increased mitochondrial localization of Bax and tBid, but also the loss of Bad phosphorylation at Ser^{112}, Ser^{136} and Ser^{155}. This was in contrast to the presence of lower amounts of Bax and tBid

at the mitochondria, and loss of Bad phosphorylation at Ser^{112} and Ser^{155}, but not at Ser^{136} seen in the absence of JNK inhibition. In its unphosphorylated form Bad interacts with Bcl-2 or Bcl-X_L (Bae, Hsu et al. 2001) and dephosphorylation of all three Ser moieties is essential for the ability of Bad to sequester Bcl-2 and Bcl-X_L (Datta, Ranger et al. 2002; Bae and Song 2003) and to facilitate tBid-induced molecular interaction between the proapoptotic partners Bax and Bak. Ser^{155} phosphorylation is known to promote the dissociation of Bad from Bcl-X_L, (Tan, Demeter et al. 2000) suggesting that p38α and/or p38β may influence mitochondrial dysfunction by regulating the molecular interaction between pro- and anti-apoptotic Bcl-2 proteins. Direct regulation of Bad phosphorylation by p38 and JNK has not been shown. Therefore, it may be mediated via other kinases including p21-activated kinase, PKA, PKC, Raf-1, Rsk and Akt/PKB (Schurmann, Mooney et al. 2000; Gnesutta, Qu et al. 2001; Wolf, Witte et al. 2001; Jones, Elford et al. 2002) and phosphatases such as PP1, PP2A and PP2B (Ayllon, Martinez et al. 2000; Chiang, Harris et al. 2001). The manner in which these enzymes are functionally modulated by 38α, p38β and JNK remains to be elucidated.

Fas-induced targeting of Bax and tBid and the loss of phosphorylation at Ser^{112} and Ser^{155} in Bad were prevented by the inhibition of p38 irrespective of the presence or absence of JNK activity. The ability of JNK inhibitor to sensitize mitochondrial proapoptotic activity was seen in si-p38β, but not si-p38α cells. Moreover, the degree of sensitizing effect of JNK inhibition was significantly lower in si-p38β cells compared to that seen in si-JNK1/2 or si-C cells (Fig. 9). The stimulatory effect of p38α and the inhibitory effect of p38β on Fas-mediated caspase-8 activation largely account for the observed differences in the proapoptotic events distal to caspase-8. As expected, Fas-induced mitochondrial presence of tBid and Bax, and loss of

phosphorylation of Ser^{70} in Bcl-2 and the phosphorylation of Ser^{112} and Ser^{155} in Bad were not seen in si-p38α cells. Likewise, in si-p38β cells, which were more sensitive to Fas-signaling due to the presence and activation of p38α, I observed the expected presence of tBid at the mitochondria, reduction in $\Delta\Psi m$, cytochrome c release and, activation of caspase-9 and caspase-3. However, Bax was excluded from the mitochondria despite the presence of tBid at this organelle in si-p38β cells. The failure of Bax to localize to the mitochondria despite the presence of tBid in cells lacking p38β may, therefore, be due to the lack of Bad dephosphorylation at Ser^{112} and Ser^{155} (Yusta, Boushey et al. 2000). An additional mechanism may involve Bcl-2 dependence of Bax recruitment. Such a regulation has been reported to result from altered Bcl-2 phosphorylation (Ishikawa, Kusaka et al. 2003). Absence of Bax at the mitochondria correlated with reduced Bcl-2 (Ser^{70}) phosphorylation in cells treated with PD169316 and in si-p38α, but not si-p38β cells. Hence p38α-dependent phosphorylation of Bcl-2 may be necessary to facilitate mitochondrial localization of Bax in cells undergoing apoptosis. Recently p38 was shown to be sequestered in the mitochondria in apoptotic cells, raising the possibility that it may directly regulate the disruption of this organelle (Tikhomirov and Carpenter 2004). The intriguing possibility that p38 may affect the mitochondrial integrity in an isoform-specific manner through event(s) that are independent of pro- and anti-apoptotic Bcl-2 proteins remains to be tested.

PD169316 treatment enhanced Fas-mediated apoptosis more than 5-fold from 5.4% to 30% in si-p38α cells and by 2.5-fold from 12% to 30.5% in si-C cells. By contrast, its effect was inhibitory in si-p38β and si-JNK1/2 cells. Caspase-8 activation was unaffected by PD169316 in si-JNK1/2 cells, enhanced in si-C and si-p38α cells and inhibited in si-p38β cells. SP600125 was able to potentiate caspase-8 activation and apoptotic responsiveness in si-p38β but not in si-p38α cells.

These data indicate that in Jurkat cells both p38β and JNK inhibit caspase-8 activation at the level of DISC in a non-additive manner and their effects are offset by p38α-mediated DISC activation. Additionally, mitochondrial presence of tBid was inhibited by PD169316 despite the potentiation of caspase-8 and the cleavage of Bid into tBid in si-C and Jurkat cells and its mitochondrial presence was inhibited uniquely in Fas-activated p38α cells. Thus, its localization to the mitochondria is dependent on the presence and activation of p38α. The mechanism involved in such a regulation is not known, but may be modulated by the effect of p38α on other BH-domain bearing proteins such as Bfl1/A1, which was recently implicated in the mitochondrial targeting of tBid (Werner, de Vries et al. 2002). The present findings have thus demonstrated the opposing effects of p38α and p38β on caspase-8 activation, similar effects on Bad phosphorylation and mitochondrial targeting of Bax and p38α-dependent mitochondrial localization of tBid. Finally, my findings also reveal that p38α, but not p38β can promote the direct caspase activation cascade as evidenced by the increased activation of caspases-8 and -3 but not of caspase-9 in si-p38β cells. Such isoform-specific effects of p38α and p38β could not be discerned in studies employing the pyridylimidazole inhibitors and underscore the need to reinterpret the previously reported conclusions derived from the pharmacological inhibition of p38.

HeLa cells are known to be resistant to Fas induced apoptosis due to Fas mediated activation of ERK1/2 (Holmstrom, Tran et al. 1999). ERK1/2 exerts their antiapoptotic effect by the phosphorylation and subsequent inhibition of procaspase-9 (Allan, Morrice et al. 2003). Post-translational modification of procaspase-9 blocked Fas mediated activation of the intrinsic apoptotic pathway in HeLa cells. I have shown here that ERK activation also inhibits the

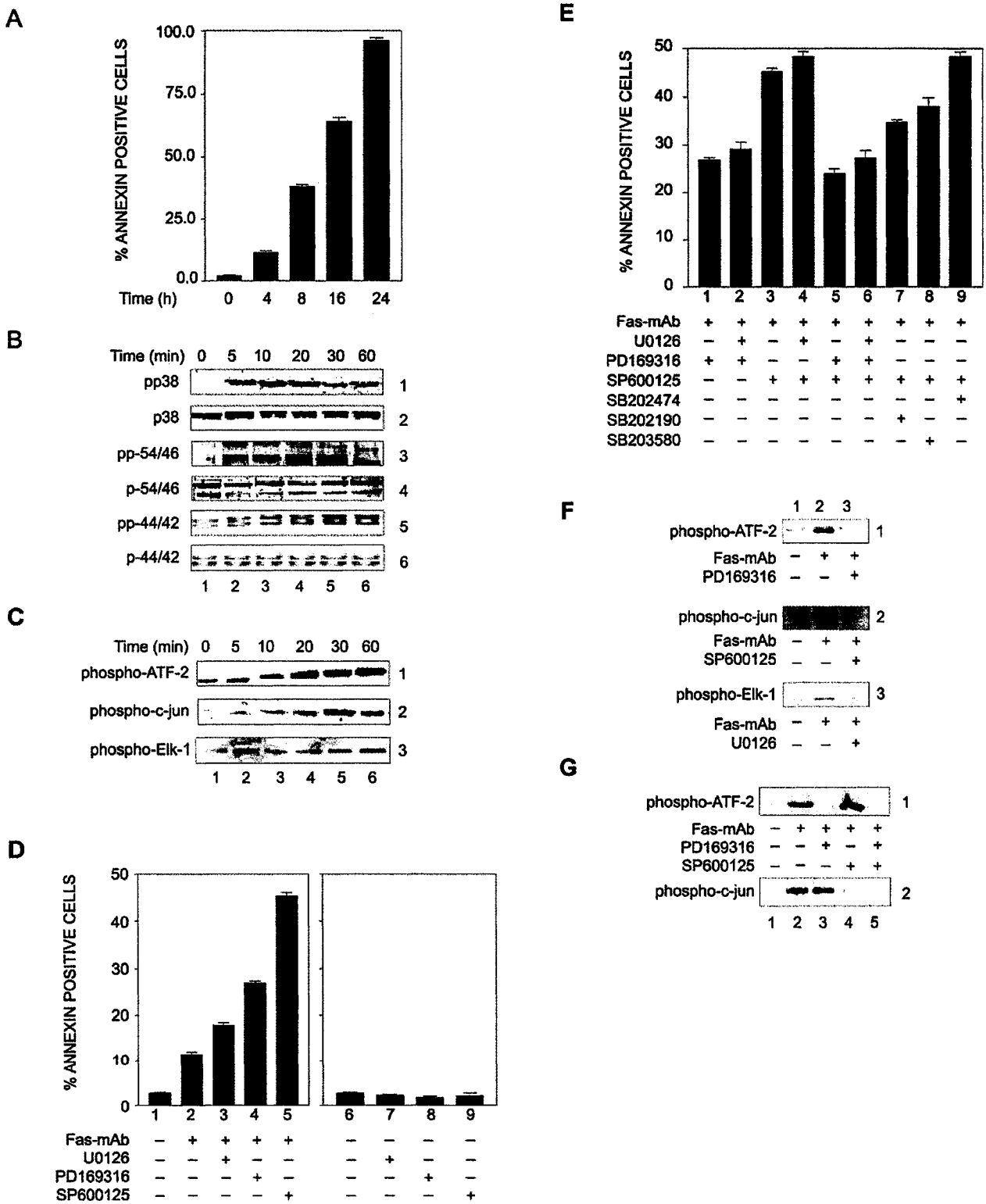
activation of pro-apoptotic p38α. These findings reveal a previously unknown mechanism of Fas induced apoptosis by ERK. Not only does ERK1/2 activation block the intrinsic pathway but also blocks the extrinsic, by inhibiting p38α mediated activation of caspase-8. This is the first evidence for isoform specific cross talk between ERK and p38α. Earlier reports suggested that inhibition of p38 using chemical inhibitors induced the activation of ERK (Berra, Diaz-Meco et al. 1998; Iwama, Nakajo et al. 2001). My findings demonstrate however that inhibition of ERK induces a sensitization of HeLa cells to Fas mediated apoptosis in a manner requiring p38 activation. This result indicates that by inhibiting anti-apoptotic ERK we activate p38α, which is a pro-apoptotic kinase. However if we inhibit p38α we decrease the level of sensitization achieved by inhibition of ERK alone. The data I have obtained thus far with HeLa cells sheds some light on the interactions between ERK and p38 axes of signaling and warrant further studies to elucidate the inter-regulatory influences of MAPKs in apoptotic signaling.

My data has clearly revealed that p38 and JNK regulate the actions of the Bcl-2 family of proteins (Fig. 7). However my findings also suggest that overexpression of anti-apoptotic Bcl-2 protein at the level of the ER but not the mitochondria abrogates Fas sensitization during p38 and JNK inhibition in Jurkat and HeLa cells. Overexpression of Bcl-2-cb5 lowered the extent of apoptosis compared to the parental cells suggesting that ER resident Bcl-2 might protect the mitochondria from upstream apoptotic signaling. Indeed it has been shown that ER resident Bcl-2 inhibits Bax translocation to the mitochondria and ER blocking Bax mediated apoptosis by blocking calcium leakages into the cytosol (Thomenius and Distelhorst 2003). These observations strengthen my finding that observation that Bcl-2 is not required to be only present at the level of the mitochondria to prevent cell death. There is a wide array of claims suggesting

that Bcl-2 function within a given organelle is not only cell type but also stimuli specific, making it difficult to unveil the specific actions of Bcl-2 at the level of the ER or the mitochondria (Rudner, Lepple-Wienhues et al. 2001; Rudner, Jendrossek et al. 2002). My data involving MAPK cross talk and organelle specific actions of Bcl-2 are of preliminary nature and emphasize the need for further studies to decipher the effects of MAPKs, in particular those of p38α and p38β on organelle-specific function of Bcl-2.

In summary, I have shown that p38α facilitates the activation of the mitochondrial arm of the apoptotic signaling at multiple levels by promoting Fas-induced caspase-8 activation, mitochondrial localization of tBid and, by directly influencing the phosphorylation status of Bad (Ser^{112} and Ser^{155}) and Bcl-2 (Ser^{70}). On the other hand, p38β controls the sensitivity of type II signaling solely by regulating Ser^{112} and Ser^{155} phosphorylation in Bad. JNK1/2 attenuates the antiapoptotic activity of Bad by inhibiting dephosphorylation of Ser^{136}. These findings suggest that selective inhibition of p38β and JNK1/2, but not p38α, might be a novel therapeutic strategy for sensitizing lymphocyte-derived tumor cells to Fas-mediated apoptosis. However this regulation is not uniform for all cell types as I show here with my data using HeLa cells. In Jurkat cells p38α was activated, allowing c-$FLIP_S$ dephosphorylation and concomitant apoptosis. During Fas treatment in HeLa cells, p38α is not activated, given the robust activation of ERK and lack of apoptosis. Lack of p38α activation allows for c-$FLIP_S$ to remain phosphorylated and bound to the DISC complex. The data presented here suggest that the pro-apoptotic nature of p38β cannot override the anti-apoptotic effect of ERK.

5.0 Figures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75.0
50.0
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B
Time (min) 0 5 10 20 30 60
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p38 2
pp-54/46 3
p-54/46 4
pp-44/42 5
p-44/42 6
1 2 3 4 5 6
C
Time (min) 0 5 10 20 30 60
phospho-ATF-2 1
phospho-c-jun 2
phospho-Elk-1 3
1 2 3 4 5 6
D
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PD169316 – – – + – – – + –
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Fas-mAb – + +
PD169316 – – +
phospho-c-jun 2
Fas-mAb – + +
SP600125 – – +
phospho-Elk-1 3
Fas-mAb – + +
U0126 – – +
G
phospho-ATF-2 1
Fas-mAb – + + + +
PD169316 – – + – +
SP600125 – – – + +
phospho-c-jun 2
1 2 3 4 5

Figure 1:

Fas treatment of Jurkat cells induces the phosphorylation and activation of p38, JNK1/2 and ERK1/2.

A. Fas induced apoptosis in Jurkat cells for up to 24 hours. B. Time dependent increase occurred in the phosphorylation of p38 (p44/42) (blot 1) JNK1/2 (blot 3) and ERK1/2 (p46/p54) (blot 5) in the absence of any change in the total levels of p38 (blot 2), JNK1/2 (blot 4) and ERK1/2 (blot 6). C. Time dependent in vitro activity of each kinase was assessed following immunoprecipitation with the respective phosphospecific antibodies. Increased phosphorylation of p38 correlated with its ability to phosphorylate ATF-2 (blot 1), JNK with its ability to phosphorylate c-jun (blot 2) and ERK with its ability to phosphorylate Elk-1 in vitro, of (blot 3). D. Jurkat cells were incubated with Fas-mAb (150 ng/ml) in the absence and presence of 15 μM U0126, PD169316 or SP600125 4 h. Fas-signaled apoptosis (lane 2) was enhanced 1.7-fold in cells treated with U0126 (lane 3), 2.8-fold in cells treated with PD169316 (lane 4) and by 4.2 fold in cells treated with SP600125 (lane 5). These inhibitors by themselves did not trigger apoptosis (lanes 7 to 9). E. Quantification of Annexin-V positive cells by flow cytometry in cells incubated with Fas-mAb in presence of the indicated combinations of MAPK inhibitors. (Mean ± SE, n=12). F. The ability of chemical inhibitors to inhibit Fas mediated activation of p38, JNK and ERK activities was accessed by in-vitro activity assay. G. Cells were incubated with Fas-mAb for 4 h in the absence (lane 2) or presence of PD169316 (lane 3), SP600125 (lane 4) or both (lane 5) and kinase activities were accessed by an in-vitro activity assay.

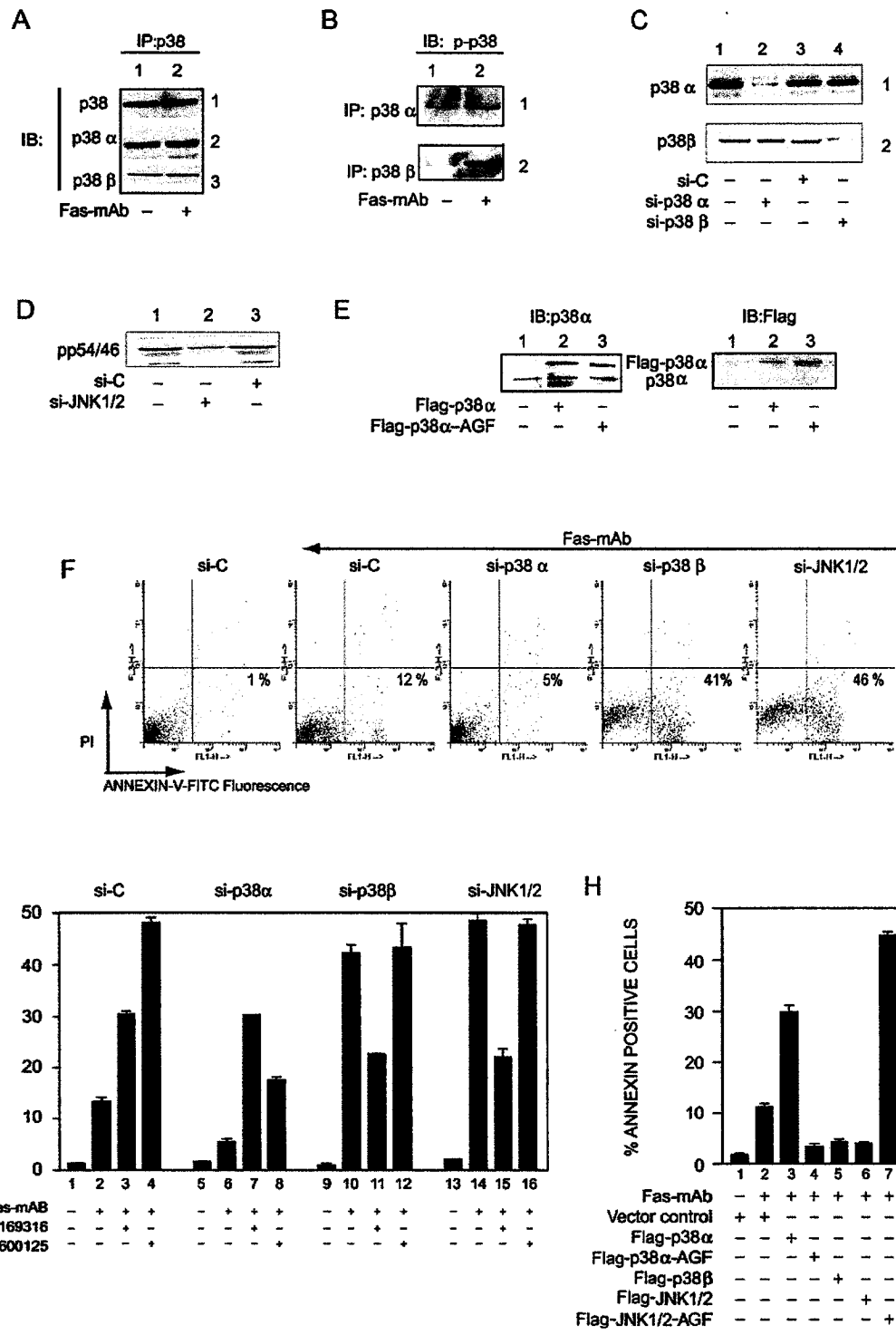

A
IP:p38
1 2
IB:
p38
p38 α
p38 β
1
2
3
Fas-mAb – +
B
IB: p-p38
1 2
IP: p38 α
IP: p38 β
1
2
Fas-mAb – +
C
1 2 3 4
p38 α
p38β
1
2
si-C – – + –
si-p38 α – + – –
si-p38 β – – – +
D
1 2 3
pp54/46
si-C – – +
si-JNK1/2 – + –
E
IB:p38α
IB:Flag
1 2 3
1 2 3
Flag-p38α
p38α
Flag-p38α – + –
Flag-p38α-AGF – – +
– + –
– – +
F
Fas-mAb
si-C
si-C
si-p38 α
si-p38 β
si-JNK1/2
1 %
12 %
5%
41%
46 %
PI
ANNEXIN-V-FITC Fluorescence
G
si-C
si-p38α
si-p38β
si-JNK1/2
% ANNEXIN POSITIVE CELLS
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fas-mAB – + + + – + + + – + + + – + + +
PD169316 – – + – – – + – – – + – – – + –
SP600125 – – – + – – – + – – – + – – – +
H
% ANNEXIN POSITIVE CELLS
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12
Fas-mAb – + + + + + + – – – – –
Vector control + + – – – – – – – – – –
Flag-p38α – – + – – – – + – – – –
Flag-p38α-AGF – – – + – – – – + – – –
Flag-p38β – – – – + – – – – + – –
Flag-JNK1/2 – – – – – + – – – – + –
Flag-JNK1/2-AGF – – – – – – + – – – – +

Figure 2:

p38α and p38β differentially influence Fas mediated cell death in Jurkat cells.

A. p38 was immunoprecipitated using a pan anti-p38 antibody (Blot 1) and total levels of p38α (Blot 2) and p38β (Blot 3) were evaluated by immunoblot analysis. B. Fas induced phosphorylation of p38α and p38β was detected by immunoprecipitating with antibodies specific for p38α (blot 1) or p38β (blot 2) and immunoblotted using phospho-specific anti-p-38 antibody. C. Inhibition of p38α, p38β and JNK using si-RNA mediated translational silencing. Jurkat cells were transfected with control si-RNA (si-C), si-RNA targeted against p38α (si-p38α) or p38β (si-p38β). D. Expression of JNK1 and JNK2 were decreased in si-JNK1/2 cells. E. Inhibition of p38α via overexpression of wild type and inactive mutant variants of p38α. Immunoblot analysis using anti-p38α antibody (left panel) and anti-Flag antibody (right panel) demonstrate the expression of transiently transfected Flag-p38α and Flag-p38α-AGF. F. Fas-induced apoptosis was higher in si-p38β and si-JNK1/2 cells, but lower in si-p38α cells compared to that in si-C cells is evident from the increase in Annexin-V-FITC positive, PI negative cells (lower right quadrants). G. PD169316's effect on SP600125-induced sensitization of Fas-mediated apoptosis in cells lacking p38α (si-p38α), p38β (si-p38β) and JNK1/2 (si-JNK1/2). H. Cells over-expressing wild type p38α (FLAG-p38α), p38β (FLAG-p38β) and JNK1/2 (FLAG-JNK1/2) or dominant negative variant of p38α (FLAG-p38α-AGF) and JNK1/2 (FLAG-JNK1/2-AGF) were treated with Fas m-Ab and analyzed for annexin positive cells by FACS. (Mean ± SE, n=12)

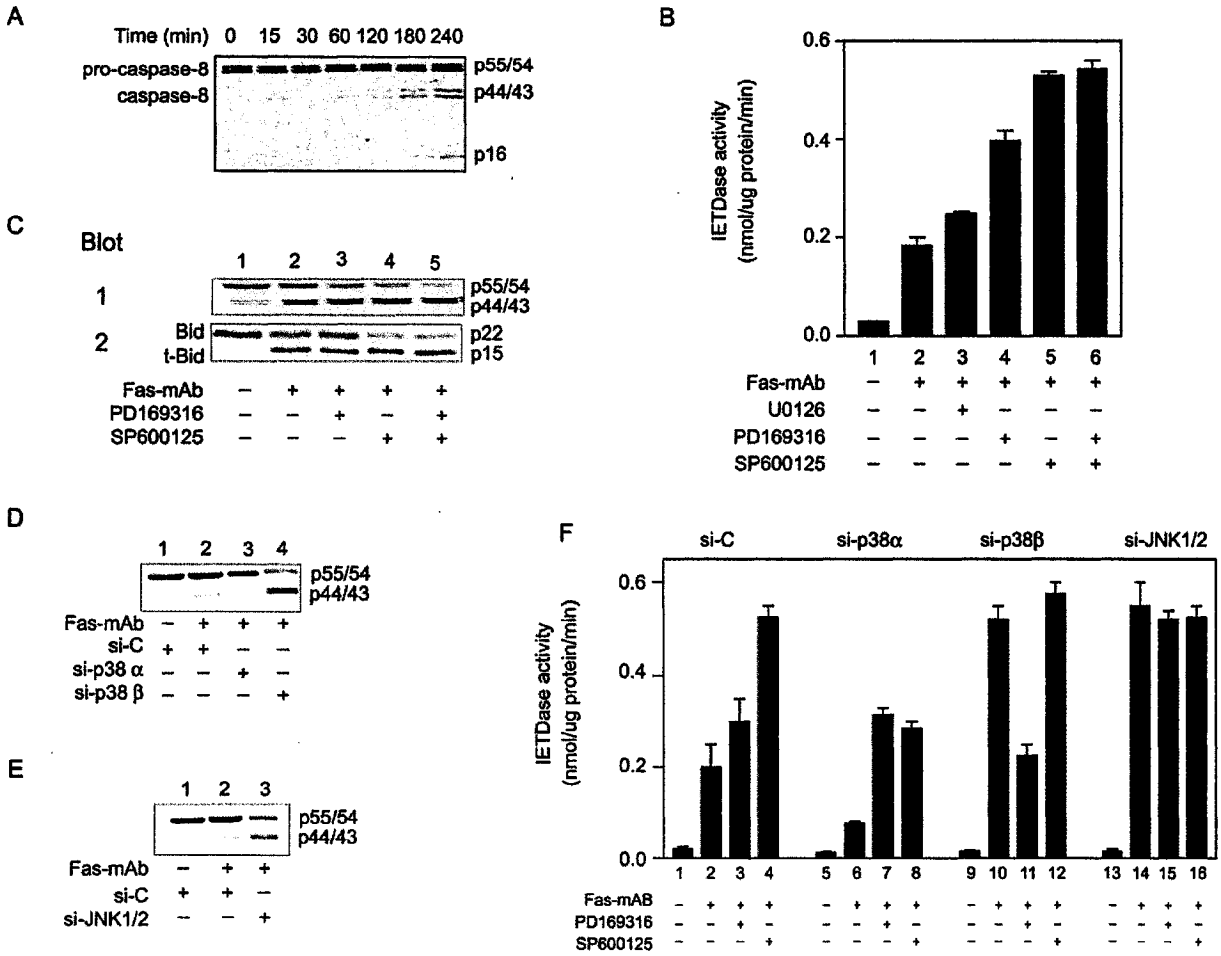

A
Time (min) 0 15 30 60 120 180 240
pro-caspase-8
caspase-8
p55/54
p44/43
p16
B
IETDase activity (nmol/ug protein/min)
0.6
0.4
0.2
0.0
1 2 3 4 5 6
Fas-mAb – + + + + +
U0126 – – + – – –
PD169316 – – – + – +
SP600125 – – – – + +
C
Blot
1 2 3 4 5
1
p55/54
p44/43
2
Bid
t-Bid
p22
p15
Fas-mAb – + + + +
PD169316 – – + – +
SP600125 – – – + +
D
1 2 3 4
p55/54
p44/43
Fas-mAb – + + +
si-C + + – –
si-p38 α – – + –
si-p38 β – – – +
E
1 2 3
p55/54
p44/43
Fas-mAb – + +
si-C + + –
si-JNK1/2 – – +
F
si-C
si-p38α
si-p38β
si-JNK1/2
IETDase activity (nmol/ug protein/min)
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fas-mAB – + + + – + + + – + + + – + + +
PD169316 – – + – – – + – – – + – – – + –
SP600125 – – – + – – – + – – – + – – – +

Figure 3:

Relationship between Fas mediated changes of MAPK and caspase-8 activation.

A. Cells were incubated with the agonistic anti-Fas antibody in a time course experiment for up to 4 hours and aliquots of cell lysates containing equal amounts of protein were analyzed for caspase activation. Immunoblot analysis shows the presence of cleaved forms of caspase-8 were evident by 2 hours of Fas ligation. B. Caspase activities were measured, in extracts of cells treated with Fas for 3 h, using the caspase-8 substrate IETD-AMC in presence of a caspase-3 inhibitor (Ac-DEVD-CHO). C. Immunoblot analysis demonstrating the effects of PD169316 and SP600125 on the formation of cleaved fragments of caspase-8 (blot 1). The lack of a difference in the extent of cleavage of caspase-8 in the presence of both inhibitors is confirmed by immunoblot analysis of its substrate Bid which is cleaved into tBid (blot 2). D & E. Fas-induced cleavage of procaspase-8 was evident in si-C cells and was inhibited in si-p38α cell but enhanced in si-p38β cells (D) and in si-JNK cells (E). F. Fas-mediated activation of caspase-8 in si-p38α cells was observed in presence, but not in absence of PD169316 and/or SP600125.

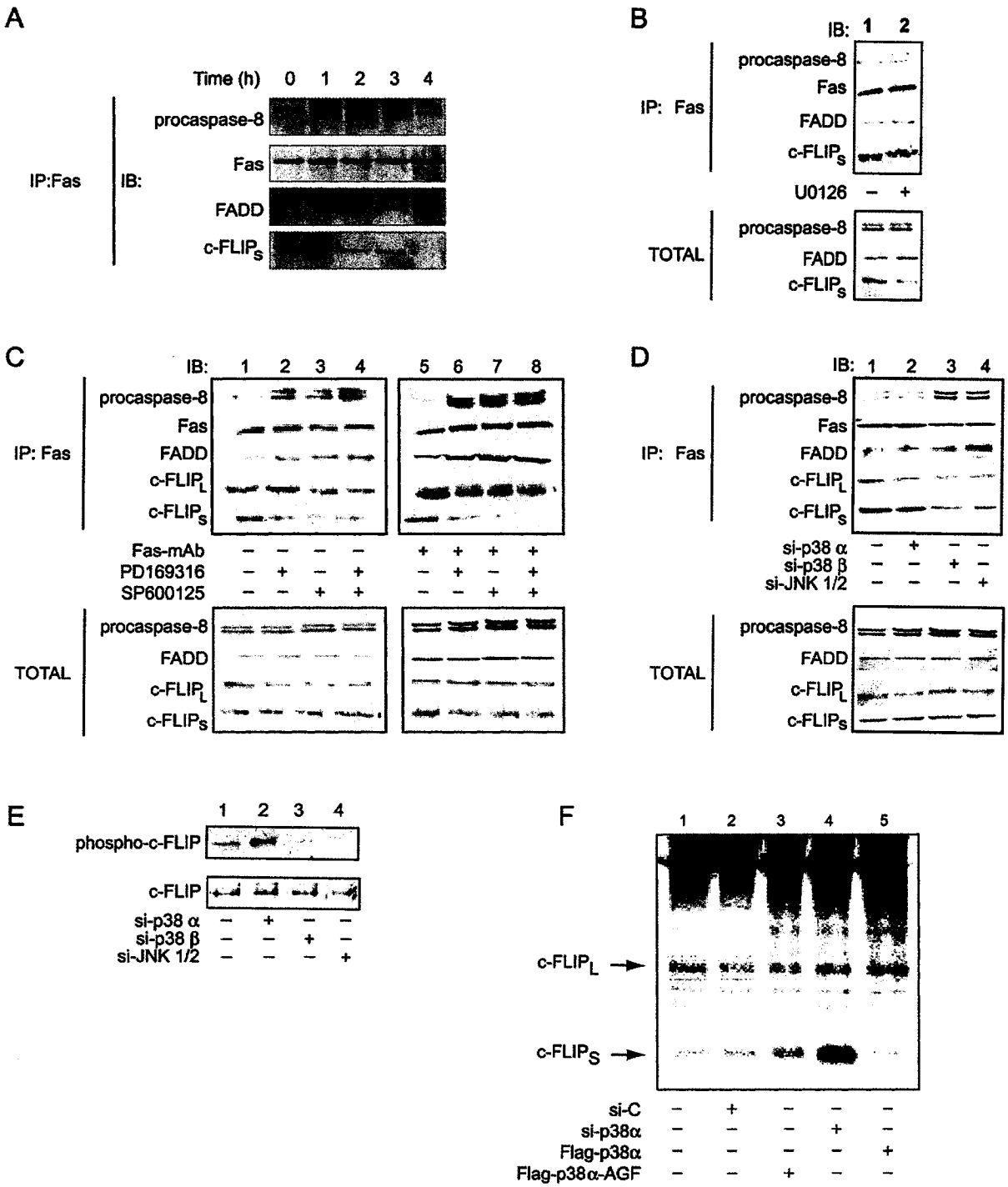
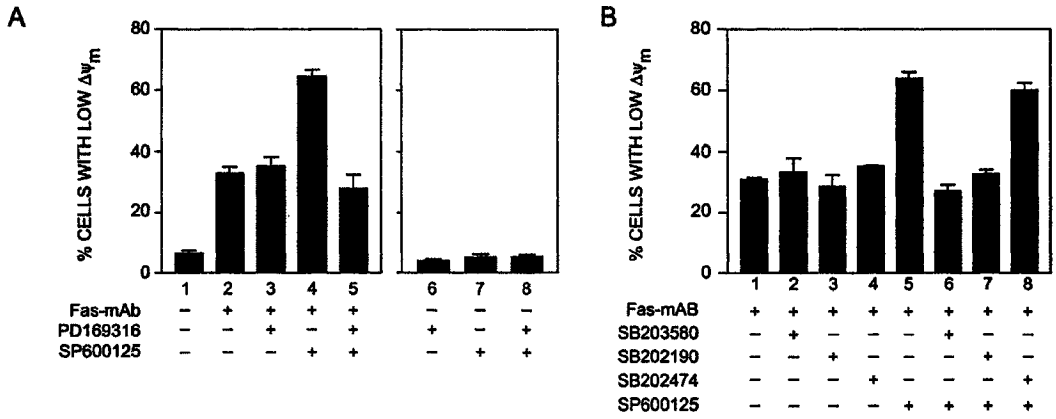
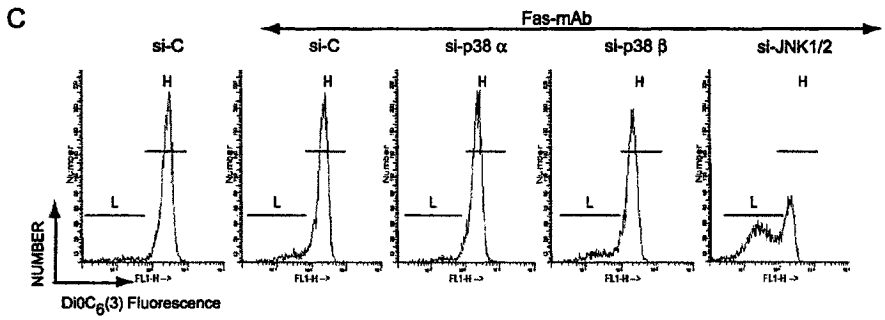

A
Time (h) 0 1 2 3 4
IP:Fas
IB:
procaspase-8
Fas
FADD
c-FLIPS
B
IB: 1 2
IP: Fas
procaspase-8
Fas
FADD
c-FLIPS
U0126 – +
TOTAL
procaspase-8
FADD
c-FLIPS
C
IB: 1 2 3 4 5 6 7 8
IP: Fas
procaspase-8
Fas
FADD
c-FLIPL
c-FLIPS
Fas-mAb – – – – + + + +
PD169316 – + – + – + – +
SP600125 – – + + – – + +
TOTAL
procaspase-8
FADD
c-FLIPL
c-FLIPS
D
IB: 1 2 3 4
IP: Fas
procaspase-8
Fas
FADD
c-FLIPL
c-FLIPS
si-p38 α – + – –
si-p38 β – – + –
si-JNK 1/2 – – – +
TOTAL
procaspase-8
FADD
c-FLIPL
c-FLIPS
E
1 2 3 4
phospho-c-FLIP
c-FLIP
si-p38 α – + – –
si-p38 β – – + –
si-JNK 1/2 – – – +
F
1 2 3 4 5
c-FLIPL
c-FLIPS
si-C – + – – –
si-p38α – – – + –
Flag-p38α – – – – +
Flag-p38α-AGF – – + – –

Figure 4:

MAPK inhibition and DISC assembly: Decreased levels of p38α induce increased presence and phosphorylation of c-FLIP$_S$ but not c-FLIP$_L$ within the DISC.

A. Lysates of Fas treated cells for up to 4 hours were immunoprecipitated with non-agonistic anti-Fas antibody and analyzed for the presence of Fas-associated proteins. B. Treatment of Jurkat cells with U0126 in the absence of Fas does not alter the composition of DISC. C. Top panel: The increased presence of FADD in the DISC and the concomitant decrease in the presence of c-FLIP$_S$ but no c-FLIP$_L$ is seen in PD169316-treated native cells (lane 2) and Fas-activated cells (lane 6). Similar changes were observed in presence of SP600125 in the native (lane 3) and Fas-activated (lane 7) cells. The effects of these inhibitors did not appear to be synergistic or antagonistic in the absence (lane 4) or Fas-activation (lane 8). Bottom Panel: Total levels of DISC components remained unchanged in the absence (left panel) and presence (right panel) of Fas. D. Presence of c-FLIP in DISC is inhibited while that of FADD and procaspase-8 is increased in si-p38β and si-JNK1/2 cells, but not in si-p38α cells (top panel) in the absence any change in protein expression (bottom panel). E. Phosphorylation of c-FLIP$_S$ is enhanced in si-p38α, but not si-p38β or si-JNK1/2 cells (top panel). Phosphorylation of c-FLIP was determined by Pro Q diamond phosphoprotein gel stain following immunoprecipitation with anti-cFLIP antibody. No change in c-FLIP$_S$ levels was seen (bottom panel). F. Jurkat cells expressing control (si-C) or p38α si-RNA (si-p38α) and FLAG-p38α or Flag-p38α-AGF were metabolically labeled with ^{32}P-sodium orthophosphate. Phosphorylated c-FLIP was immunoprecipitated from the lysates with anti-c-FLIP antibody that recognizes both c-FLIP$_S$ and c-FLIP$_L$, electrophoresed on SDS-polyacrylamide gels and subjected to autoradiographic analysis. Data representative of 3 experiments.


A
% CELLS WITH LOW Δψm
Fas-mAb
PD169316
SP600125
B
% CELLS WITH LOW Δψm
Fas-mAB
SB203580
SB202190
SB202474
SP600125


C
Fas-mAb
si-C
si-C
si-p38 α
si-p38 β
si-JNK1/2
NUMBER
Di0C6(3) Fluorescence

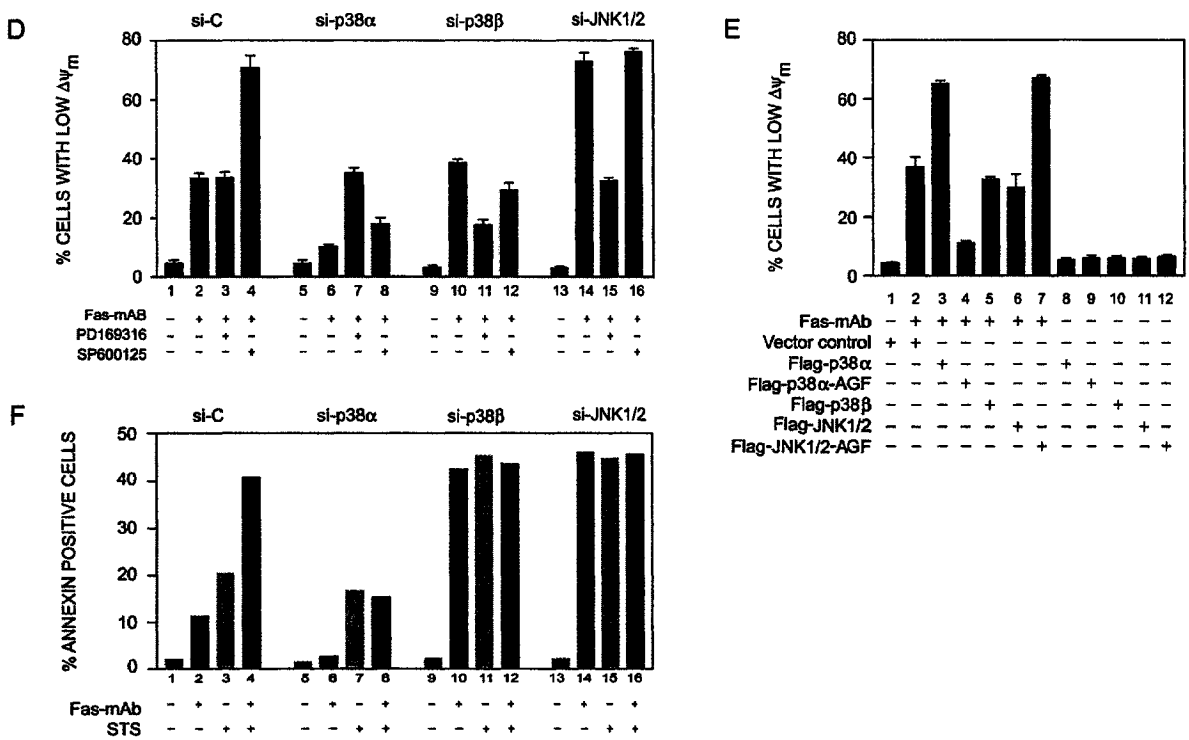
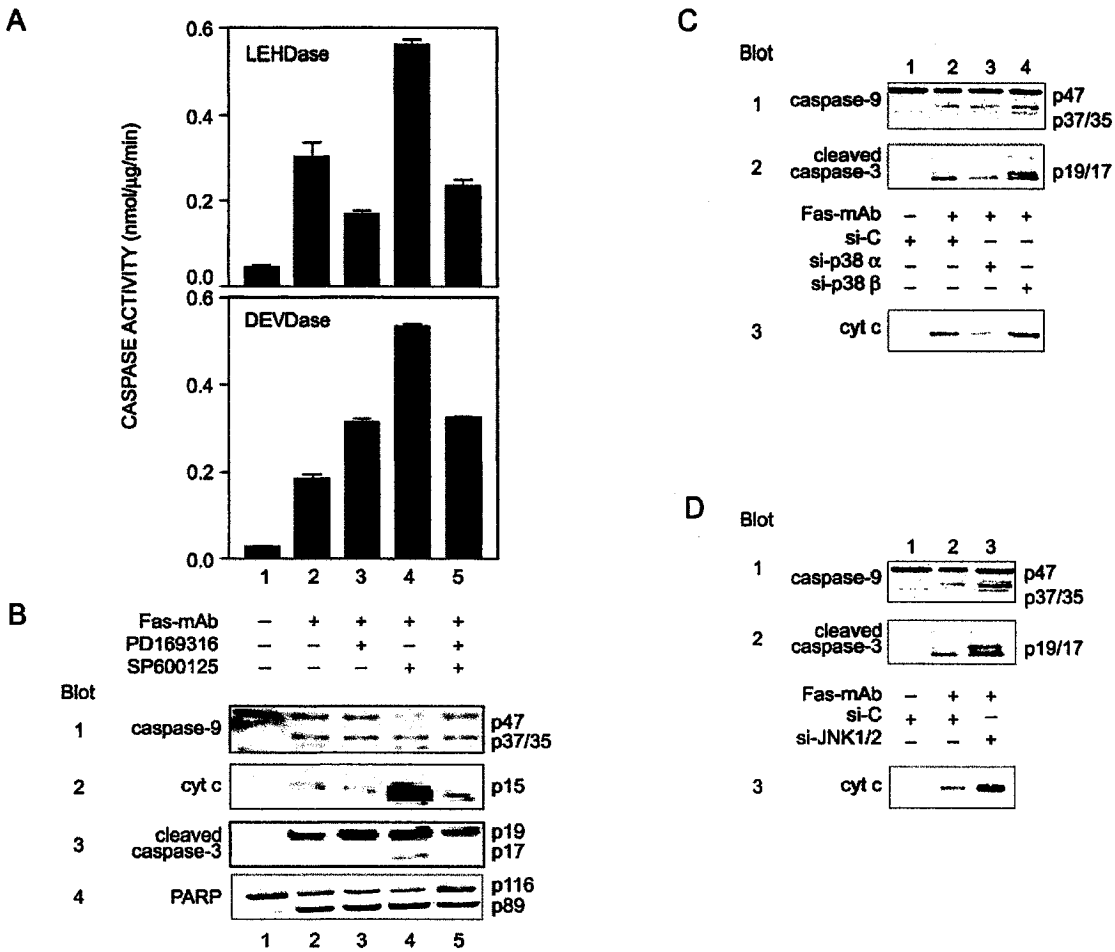

D
si-C
si-p38α
si-p38β
si-JNK1/2
% CELLS WITH LOW Δψm
Fas-mAB
PD169316
SP600125
E
% CELLS WITH LOW Δψm
Fas-mAb
Vector control
Flag-p38α
Flag-p38α-AGF
Flag-p38β
Flag-JNK1/2
Flag-JNK1/2-AGF
F
% ANNEXIN POSITIVE CELLS
Fas-mAb
STS

Figure 5:

Modulation of Fas mediated mitochondrial membrane permeability by p38 and JNK.

A. The reduction in mitochondrial membrane potential ($\Delta\psi m$) in cells undergoing Fas-mediated apoptosis (lane 2) was enhanced by SP600125 (lane 4) but not PD169316 (lane 3). The potentiation induced by SP600125 was inhibited by PD169316 (lane 5). These inhibitors by themselves (individually or together) did not trigger apoptosis (not shown) or decrease $\Delta\psi m$ (lanes 6, 7 and 8). B. The dependency of the sensitizing effect of SP600125 on Fas-mediated apoptosis was confirmed by the effects of two other p38 inhibitors SB203580 and SB202190 (compare lanes 5, 6 and 7), and by the lack of effect of nonfunctional derivative SB202474 (lane 8). Values represent mean ± SE (n=12). C. The percentage of cells with decreased $\Delta\Psi m$ (region L) compared to that of cells with high or normal $\Delta\Psi m$ (region H) was enhanced to a greater extent in si-JNK cells than in si-p38β cells, but was decreased in si-p38α cells. D. The potentiating effect of SP600125 on the reduction in $\Delta\psi_m$ in response to Fas-activation was abrogated in both si-p38α cells and si-p38β cells, but not in si-C cells. The reduction in $\Delta\psi_m$ in Fas-activated si-JNK1/2 cells was attenuated by PD169316. E. Fas-induced reduction in $\Delta\psi_m$ in Jurkat cells is potentiated by over-expression of wild type but not dominant negative Flag tagged p38α. Cells over-expressing wild type p38α (FLAG-p38α), p38β (FLAG-p38β) and JNK1/2 (FLAG-JNK1/2) or dominant negative variant of p38α (FLAG-p38α-AGF) and JNK1/2 (FLAG-JNK1/2-AGF) were treated with Fas m-Ab). F. Quantification of apoptosis of si-RNA treated cells exposed simultaneously to Fas and mitochondrial toxin staurosporine (STS). This experiment indicates that p38α (compare lanes 3 and 7) is not involved in directly inhibiting the mitochondrial activation during Fas treatment.


A
LEHDase
DEVDase
CASPASE ACTIVITY (nmol/μg/min)
0.0
0.2
0.4
0.6
1 2 3 4 5
B
Fas-mAb – + + + +
PD169316 – – + – +
SP600125 – – – + +
Blot
1 caspase-9 p47 p37/35
2 cyt c p15
3 cleaved caspase-3 p19 p17
4 PARP p116 p89
1 2 3 4 5
C
Blot
1 2 3 4
1 caspase-9 p47 p37/35
2 cleaved caspase-3 p19/17
Fas-mAb – + + +
si-C + + – –
si-p38 α – – + –
si-p38 β – – – +
3 cyt c
D
Blot
1 2 3
1 caspase-9 p47 p37/35
2 cleaved caspase-3 p19/17
Fas-mAb – + +
si-C + + –
si-JNK1/2 – – +
3 cyt c

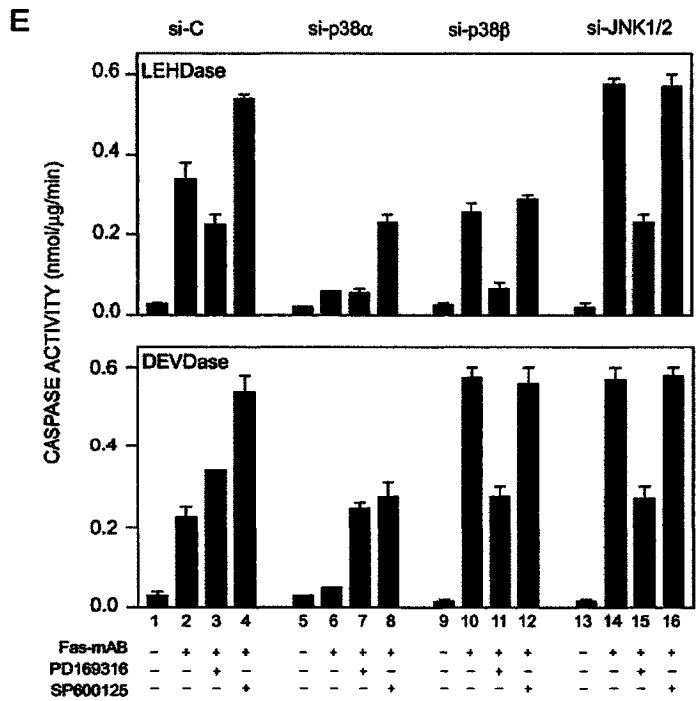
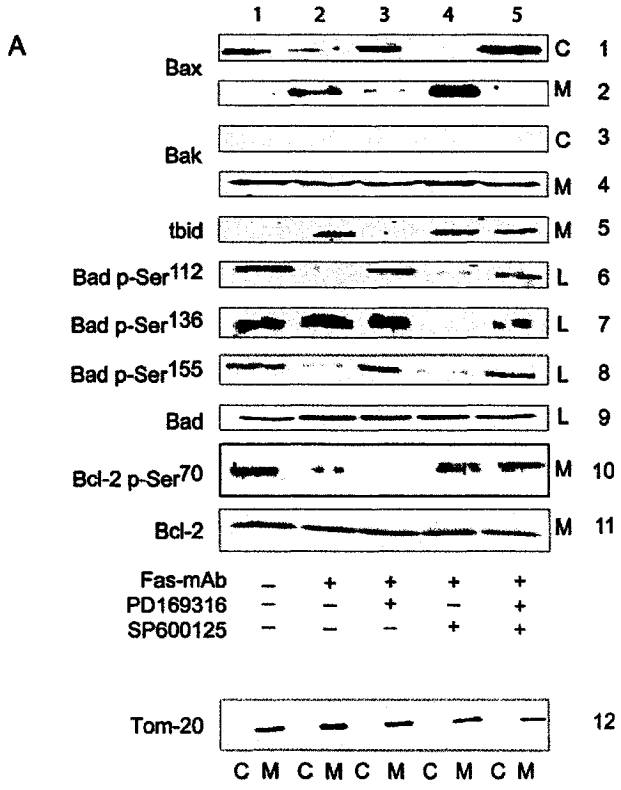

E
si-C
si-p38α
si-p38β
si-JNK1/2
LEHDase
DEVDase
CASPASE ACTIVITY (nmol/μg/min)
0.0
0.2
0.4
0.6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fas-mAB – + + + – + + + – + + + – + + +
PD169316 – – + – – – + – – – + – – – + –
SP600125 – – – + – – – + – – – + – – – +

Figure 6:

Effect of p38 and JNK inhibition on Fas mediated activation of caspase-9 and -3.

A. Caspase-9 activation (top panel) was potentiated by SP600125 (lane 4). PD169316 inhibited caspase-9 activation in Fas-activated cells both in the absence (lane 2) and in presence of SP600126 (lane 5). Caspase-3 activity (bottom panel) was higher in Fas-activated cells in presence of SP600125 (lane 4) than PD169316 (lane 3). PD169316 blunted the potentiating effect of SP600125 (lane 5). B. Immunoblot analysis demonstrating the effects of PD169316 and SP600125 on the formation of cleaved fragments of caspase-9 (blot 1) and caspase-3 (blot 3). The differential effects of these inhibitors occurring distal to caspase-8 were confirmed by the differences in cyt c release (blot 2) and PARP cleavage (blot 4). C & D. Fas-induced cleavage of procaspase-9 (blot 1) and procaspase-3 (blot 2) was evident in si-C cells and was inhibited in si-p38α cell but enhanced in si-p38β cells (compare lanes 3 and 4 with lane 2, blots 1,2,3, panel C) and in si-JNK cells (compare lanes 3 and 2, panel D). E. Fas-mediated activation of caspase-9 (top panel) and caspase-3 (bottom panel) in si-p38α cells was observed in presence, but not in absence of PD169316 and/or SP600125.


A
1 2 3 4 5
Bax
C 1
M 2
Bak
C 3
M 4
tbid
M 5
Bad p-Ser112
L 6
Bad p-Ser136
L 7
Bad p-Ser155
L 8
Bad
L 9
Bcl-2 p-Ser70
M 10
Bcl-2
M 11
Fas-mAb – + + + +
PD169316 – – + – +
SP600125 – – – + +
Tom-20
12
C M C M C M C M C M

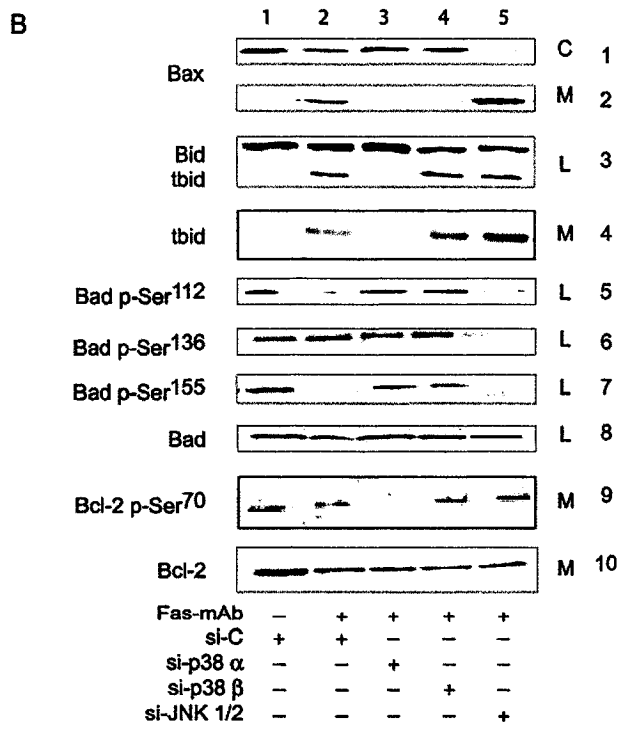

B
1 2 3 4 5
Bax
C 1
M 2
Bid
tbid
L 3
tbid
M 4
Bad p-Ser112
L 5
Bad p-Ser136
L 6
Bad p-Ser155
L 7
Bad
L 8
Bcl-2 p-Ser70
M 9
Bcl-2
M 10
Fas-mAb – + + + +
si-C + + – – –
si-p38 α – – + – –
si-p38 β – – – + –
si-JNK 1/2 – – – – +

Figure 7:

Effect of inhibition of p38 and JNK on cellular localization and/or biochemical properties of Bcl-2 family of proteins in cells undergoing Fas-mediated apoptosis.

A. Effects of PD169316 and SP600125 on proteins of the Bcl-2 family. Aliquots containing equal amounts of protein from cytosolic (C) and mitochondrial (M) fractions or whole cell lysates (L) were subjected to SDS-PAGE and immunoblot analysis. The quality of mitochondrial preparations was established by immunoblot analysis for the marker protein Tom-20 (blot 12). Data are representative of three experiments. B. Effect of si-RNA treatment on proteins of the Bcl-2 family. (Data representative of 3 experiments).

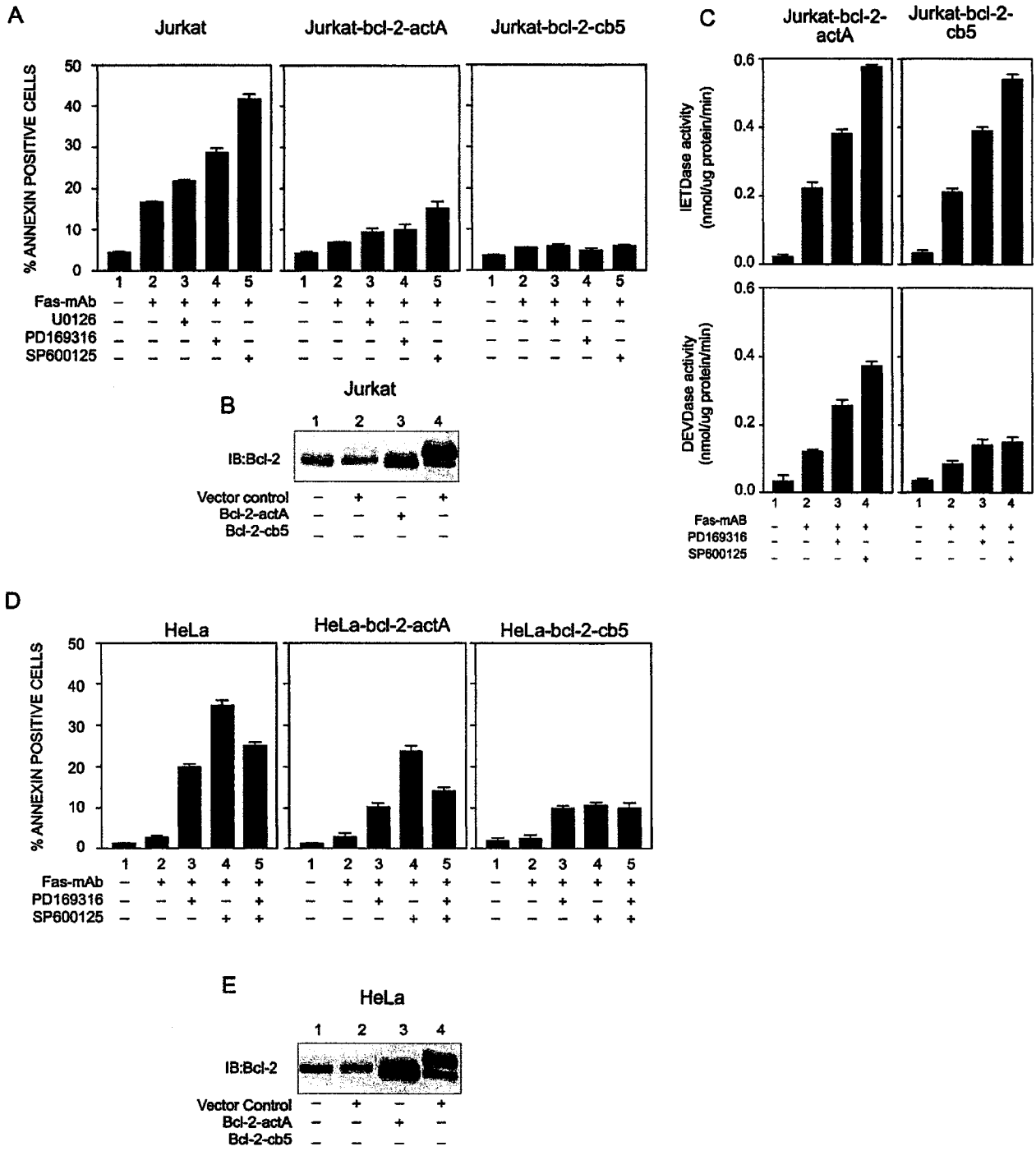
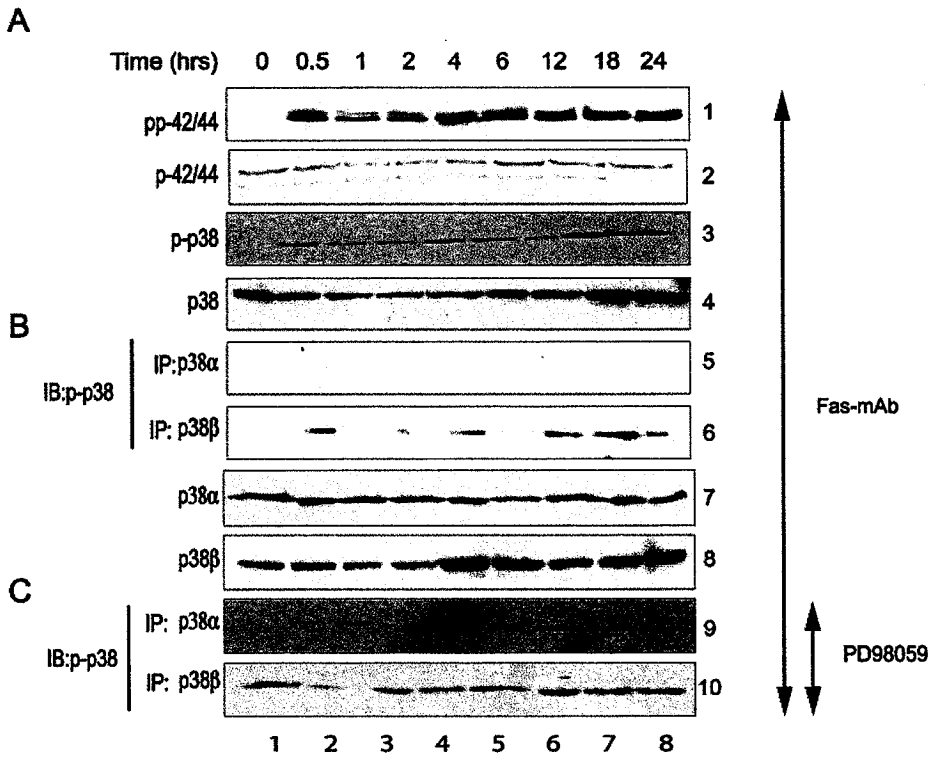
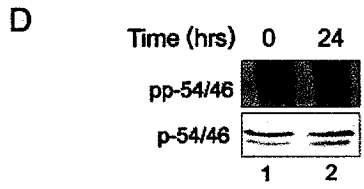

A
Jurkat
Jurkat-bcl-2-actA
Jurkat-bcl-2-cb5
% ANNEXIN POSITIVE CELLS
Fas-mAb
U0126
PD169316
SP600125
B
Jurkat
IB:Bcl-2
Vector control
Bcl-2-actA
Bcl-2-cb5
C
Jurkat-bcl-2-actA
Jurkat-bcl-2-cb5
IETDase activity (nmol/ug protein/min)
DEVDase activity (nmol/ug protein/min)
Fas-mAB
PD169316
SP600125
D
HeLa
HeLa-bcl-2-actA
HeLa-bcl-2-cb5
% ANNEXIN POSITIVE CELLS
Fas-mAb
PD169316
SP600125
E
HeLa
IB:Bcl-2
Vector Control
Bcl-2-actA
Bcl-2-cb5

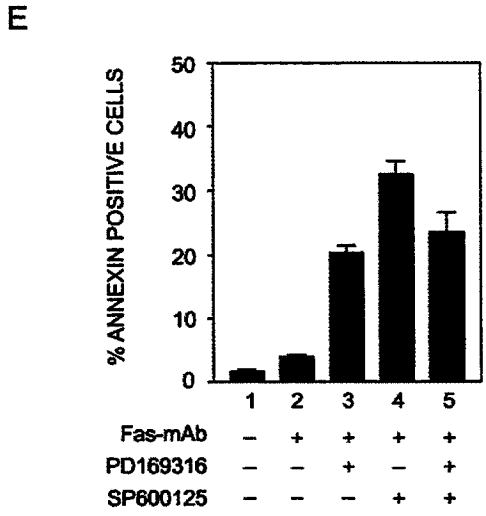
Figure 8:

ER localized Bcl-2 blocks sensitization to Fas mediated cell death during MAPK inhibition in Jurkat and HeLa cells.

A. Jurkat cells stably over-expressing Bcl-2 at the level of the mitochondria (Jurkat-blc-2-actA) and ER (Jurkat-bcl-2-cb5) were treated with Fas-mAb. Annexin positive cells were quantified and represented in graph form. B. Immunoblot analysis of Bcl-2 overexpresssion in Jurkat cells. C. Caspase-8 (top panels) and -3 (bottom panels) activity was determined in the Bcl-2 transfected Jurkat cells. (Values represent mean +/- SE, n=6) D. HeLa cells stably over-expressing Bcl-2 at the levels of the mitochondria (HeLa-blc-2-actA) and ER (HeLa-bcl-2-cb5) were treated with Fas-mAb. Annexin positive cells were quantified and represented in graph form. Compare center and right hand graph with native HeLa cells. E. Immunoblot analysis of Bcl-2 overexpresssion in HeLa cells.


A
Time (hrs) 0 0.5 1 2 4 6 12 18 24
pp-42/44
p-42/44
p-p38
p38
B
IB:p-p38
IP:p38α
IP: p38β
p38α
p38β
C
IB:p-p38
IP: p38α
IP: p38β
1 2 3 4 5 6 7 8 9 10
Fas-mAb
PD98059


D
Time (hrs) 0 24
pp-54/46
p-54/46
1 2


E
% ANNEXIN POSITIVE CELLS
0 10 20 30 40 50
1 2 3 4 5
Fas-mAb – + + + +
PD169316 – – + – +
SP600125 – – – + +

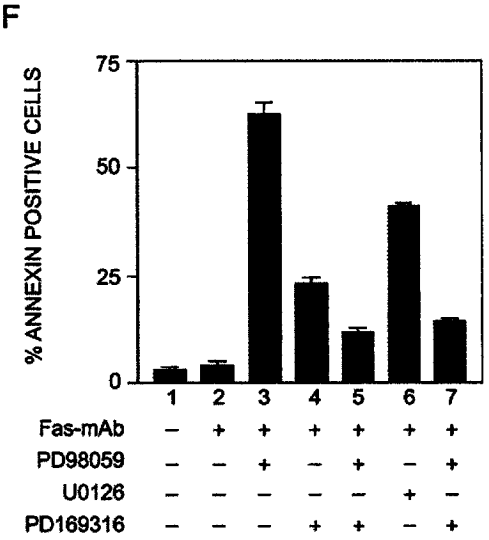

F
% ANNEXIN POSITIVE CELLS
0 25 50 75
1 2 3 4 5 6 7
Fas-mAb – + + + + + +
PD98059 – – + – + – +
U0126 – – – – – + –
PD169316 – – – + + – +

Figure 9:

Fas mediated activation of ERK1/2 inhibits pro-apoptotic p38α in HeLa cells.

A. Following incubation with Fas-mAb (150 ng/ml) HeLa cells were lysed and subjected to immunoblot analysis. Time dependent increase in of ERK1/2(p44/42) (blot 1) and p38 (blot 3) phosphorylation, in the absence of any change in the total levels of ERK (blot 2), p38 (blot 4) was observed. B. Fas induced phosphorylation of p38β but not p38α. C. Inhibition of Fas mediated ERK activation induces activation of p38α (blot 9) and p38β (blot 10) even in the absence in Fas stimulation (lane 1). D. Following incubation with Fas-mAb (150 ng/ml) HeLa cells were lysed and subjected to immunoblot analysis. Time dependent increase occurred in the phosphorylation of JNK1/2 in the absence of any change in the total levels of JNK1/2. E. Apoptosis was potentiated by PD169316 and SP600125 in Fas treated HeLa cells (lane 3 and 4 versus lane 2). Concomitant use of PD169316 and SP600125 attenuated the potentiating effect of SP600125 (lane 5). F. HeLa cells were incubated with Fas-mAb (150 ng/ml) in the absence and presence of 15 μM PD98059, U0126 and PD169316 for 24 h. Fas-signaled apoptosis (lane 2) was enhanced 60-fold in cells treated with PD98059 (lane 3), 20-fold in cells treated with PD169316 (lane 4) and by 40-fold in cells treated with U0126 (lane 6). PD169316 reduces PD98059 (lane 5) or U0126 (lane 7) mediated sensitization to Fas mediated cell death in HeLa cells.

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