rabbit serum. (d) Serum starved-HeLa cells were treated with PMA or TNFα and lysed at the indicated times. IKK and NAK activities were determined by immunecomplex kinase assays using GST-IκBα or GST-IKKβ as substrates. (e) Serum starved-A172 cells were treated with 100 ng/ml PDGF in the presence or absence of Ro 31-8220 (5 μM), and lysed at the indicated times. NAK activity was determined by immunecomplex kinase assay. (f) Increasing amounts of NAK(KM)(2.0, 3.0, and 4.0 μg/plate) were transfected with NF-κB-Luc and actin-β gal reporters into A172 cells and, 30 hours after transfection, cells were treated with PDGF for additional 6h. Luciferase activity was determined as described above.

a

NAK   1
IKK-i   1
IKKα   1
IKKβ   1

NAK   55
IKK-i 55
IKKα   61
IKKβ   61

NAK   111
IKK-i 111
IKKα 120
IKKβ 121

NAK   171
IKK-i 171
IKKα 179
IKKβ 180

NAK   231
IKK-i 231
IKKα 228
IKKβ 229

NAK   289
IKK-i 289
IKKα 286
IKKβ 287

NAK   344
IKK-i 344
IKKα 346
IKKβ 345

NAK   393
IKK-i 393
IKKα 401
IKKβ 104

NAK   449
IKK-i 449
IKKα 461
IKKβ 464

NAK   508
IKK-i 509
IKKα 521
IKKβ 524

NAK   566
IKK-i 557
IKKα 571
IKKβ 574

NAK   621
IKK-i 612
IKKα 630
IKKβ 634

NAK   673
IKK-i 665
IKKα 690
IKKβ 689

NAK   726 VDCL----
IKK-i   --------
IKKα   --------
IKKβ 749 HSCLEQAS

b

NAK

β-actin

Heart Brain Placenta Lung Liver Skeletal muscle Kidney Pancreas Spleen Thymus Prostate Testis Ovary Small intestine Colon PBL

← 2.4 kb

← 1.35 kb

a

IP   IB   1 2 3 4 5 6 7 8 9 10 11

α-Flag → Flag-IKKβ / Flag-NAK → Flag-FWD1

25 % Input

α-Myc → Myc-IκBα

α-P-IκBα (32S specific) → Myc-IκBα P-Ser32

α-Myc

α-Flag → Flag-FWD1

α-Ub → Multi-Ub IκBα

	1	2	3	4	5	6	7	8	9	10	11
NAK	+	-	+	-	-	+	+	-	-	+	-
NAK(KM)	-	-	-	-	-	-	-	+	-	-	+
IKKβ	+	-	-	+	-	+	-	-	+	+	+
FWD1	+	-	-	-	+	-	+	+	+	+	+
IκBα	-	+	+	+	+	+	+	+	+	+	+

b

anti-Flag   anti-p65/Rel A   Hoechst 33258

c

NAK	+	+	+	+	+	+
IKKβ(KM)	-	0.4	0.8	1.2	1.6	2.0
FWD1	+	+	+	+	+	+
IκBα	+	+	+	+	+	+

α-P-IκBα (32S specific) → Myc-IκBα P-Ser32

α-Flag → Flag-IKKβ / Flag-NAK

α-Myc → Myc-IκBα

d

NAK   N Wt KM Wt Wt Wt N Wt KM Wt Wt

	Wt	+	+	+								
GST-IκBα (1-54)	S32A/S36A				+							
	S32A					+						
	S36A						+					
GST-IKKβ (132-206)	Wt							+	+	+		
	S177A/S181A										+	
GST												+

   1 2 3 4 5 6 7 8 9 10 11

NAK →

GST-IKKβ / GST-IκBα →   KA

GST-IKKβ / GST-IκBα →
GST →   CBB

e

   1 2 3 4

GST-IκBα S36A →   KA

GST-IκBα S36A →   CBB

Flag-NAK →   IB

Myc-IKKβ →   IB

Flag-NAK	-	+	+	-
Flag-NAK(KM)	-	-	-	+
Myc-IKKβ	+	-	+	+

f

NAK   NAK(KM)   CONTROL

KA → GST-IκBα FL(S36A)

IB → IKKα

RA   9   1   1

g

None   Vector   NAK   NAK(KM)   Positive Control

p38

JNK

a

Relative Luciferase activity

Vector | IKKβ(0.1) | IKKβ(0.5) | NAK | IKKβ(KM) | NAK(KM) | IKKβ(0.1)+NAK

d

NAK

Relative Luciferase activity

Wt | IKKα−/− | IKKβ−/−

TNFα

Relative Luciferase activity

Wt | IKKα−/− | IKKβ−/−

b

Mut-κB
Wt-κB

None | Vector | NAK | NAK | NAK+ wt oligo | NAK+ mut oligo | NAK+ αRelA Ab | NAK(KM) | IKKβ 0.2 | IKKβ 2.0 | IKKβ(KM) | IKKβ 0.2+ NAK

NF-κB
ns

free probe

e

Relative Luciferase activity

PMA − + + + + + + +

NAK(KM)   IKKβ(KM)

Relative Luciferase activity

TNFα − + + + + + + +

NAK(KM)   IKKβ(KM)

f

Relative Luciferase activity

PMA − + + + +

NAK(KM)

c

Relative Luciferase activity

Vector | IKKβ | NAK

NAK(KM)   IKKβ(KM)

g

Relative Luciferase activity

Vector | PKC α | PKC θ | PKC ε

NAK(KM)   NAK(KM)   NAK(KM)

a

IP: NRS | αNAK

Wt | Wt | S32A/S36A | S32A | S36A

GST-IκBα ► (1-54) KA

GST-IκBα ► (1-54) CBB

b

TNF: − +

L | P | P | L

IB

NAK

IKKα

IKKβ

IKKγ

c

PMA: − +

IP: NRS NAK IKKα IKKβ IKKγ | NRS NAK IKKα IKKβ IKKγ

IB

NAK ◄ 84kDa

IKKα ◄ 85kDa

IKKβ ◄ 87kDa

d

PMA | TNFα

0 5 10 30 60 | 0 5 10 30 60 min

KA ┌ IKKα

◄ GST-IκBα (1-54)

└ NAK

◄ GST-IKKβ (132-206)

IB NAK ◄ NAK

e

Ro 31-8220 PDGF: 0 10 30 60 120 min

− ┌ KA ◄ GST-IKKβ (132-206)

└ IB ◄ NAK

+ ┌ KA ◄ GST-IKKβ (132-206)

└ IB ◄ NAK

f

PDGF − + + + +

NAK(KM)