The *Drosophila* proteins Pelle and Tube induce JNK/AP-1 activity in mammalian cells

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Abstract The mammalian interleukin-1 (IL-1) signal transduction pathways display remarkable homology to the Toll signaling cascade in *Drosophila*. To address the question whether members of the *Drosophila* Toll pathway are functional in mammalian cells, inactive and constitutively active versions of the protein kinase Pelle and its regulator Tube were expressed in HeLa cells and tested for their impact on IL-1-dependent signaling events. The *Drosophila* proteins failed to induce the IL-1-responsive transcription factor, nuclear factor-κB, but selectively activated the IL-1-regulated kinase, c-Jun N-terminal kinase (JNK), thus resulting in elevated AP-1 activity. Activation of JNK/AP-1 activity was seen upon expression of a Pelle mutant lacking its C-terminal half or by a membrane-bound and multimerised Tube protein, showing the functionality of the *Drosophila* proteins in mammalian cells.

Key words: Interleukin-1 signaling; Pelle; Tube; c-Jun N-terminal kinase; AP-1; Nuclear factor-κB

1. Introduction

The pro-inflammatory cytokine interleukin-1 (IL-1) signals via the IL-1 receptor (IL-1R), thus leading to the activation of various effector pathways including induction of transcription factor, nuclear factor (NF)-κB, and the mitogen-activated protein kinases (MAPKs), p38 and c-Jun N-terminal kinase (JNK) [1]. The large family of IL-1Rs includes the IL-1 accessory protein (IL-1AcP) which is unable to bind to the extracellular ligand, but heterodimerises with the IL-1R in the presence of IL-1. This interaction creates a signal transduction platform and allows subsequent binding of the adapter protein MyD88 [2,3], which contains an N-terminal protein/protein interaction domain, the so-called death domain (DD) [4]. The DD contained in MyD88 mediates homotypic binding to another DD contained in the serine/threonine kinase IRAK ('IL-1R-associated kinase') proteins [5]. Interestingly, the kinase function of IRAK is dispensable for its ability to transmit IL-1R-derived signals [6]. Recruitment of IRAK to the DD of MyD88 results in the autophosphorylation of IRAK and is regulated by Tollip, which inducibly associates with IL-1AcP [7]. Autophosphorylated IRAK proteins dissociate from the receptor complex and bind to TRAF-6, another DD-containing protein lacking any known enzymatic activity. Induced oligomerisation of TRAF-6 is sufficient for activation of NF-κB and JNK [8]. TRAF-6 binds to ECSIT and thereby stimulates the activity of MAPK kinase kinase (MAP3K) MEKK1, which in turn activates NF-κB as well as JNK [9]. The TRAF-6-mediated activation of the MAP3K TAK and its coactivator TAB results in the activation of IκB kinases and the induced phosphorylation and degradation of IκB, thus leading to the activation of NF-κB.

The IL-1 signaling cascade displays a striking degree of similarity to proteins establishing the dorsal/ventral polarity and host defense in *Drosophila*, as schematically shown in Fig. 1. While the *Drosophila* protein Spätzle is functionally homologous to IL-1, the IL-1R displays similarity to the *Drosophila* Toll receptor [10,11]. The Toll receptor-derived signals are further transmitted by the adapter protein Tube and its ligand Pelle, a serine/threonine kinase with homology to the vertebrate IRAK proteins [12]. The C-terminus of Pelle can be bound by Pellino [13], but the regulatory consequences of this protein/protein interaction are not yet clear. Pelle-transmitted signals are transmitted by so-far unknown intermediates to Cactus, an IκB homologue. The homology extends to the *Drosophila* protein Dorsal, which corresponds to NF-κB. In addition to its role in dorsoventral pattern formation, Dorsal, together with its homologue Dif, is also important for the antifungal response [14]. The *Drosophila* protein Relish is a homologue of the mammalian p105 precursor of the NF-κB p50 protein and is required for the antibacterial immune response in the fly [15].

The parallels between the IL-1 system in vertebrates and the dorsal signaling pathways in *Drosophila* suggest that the characterisation of genes involved in the fly system will provide general information about the regulation of IL-1-derived activation cascades. To test whether the *Drosophila* proteins Pelle and Tube function in IL-1-regulated signaling cascades in vertebrate cells, we expressed various active and inactive forms of both fly proteins in HeLa cells and analysed their impact on NF-κB and JNK/AP-1 signaling.

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Abbreviations: IL-1, interleukin-1; IL-1R, IL-1 receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IL-1AcP, IL-1 accessory protein; DD, death domain; IRAK, IL-1 receptor-associated kinase; MAP3K, MAPK kinase kinase; DJNK, *Drosophila* JNK protein; DTRAF-1, *Drosophila* TRAF-1

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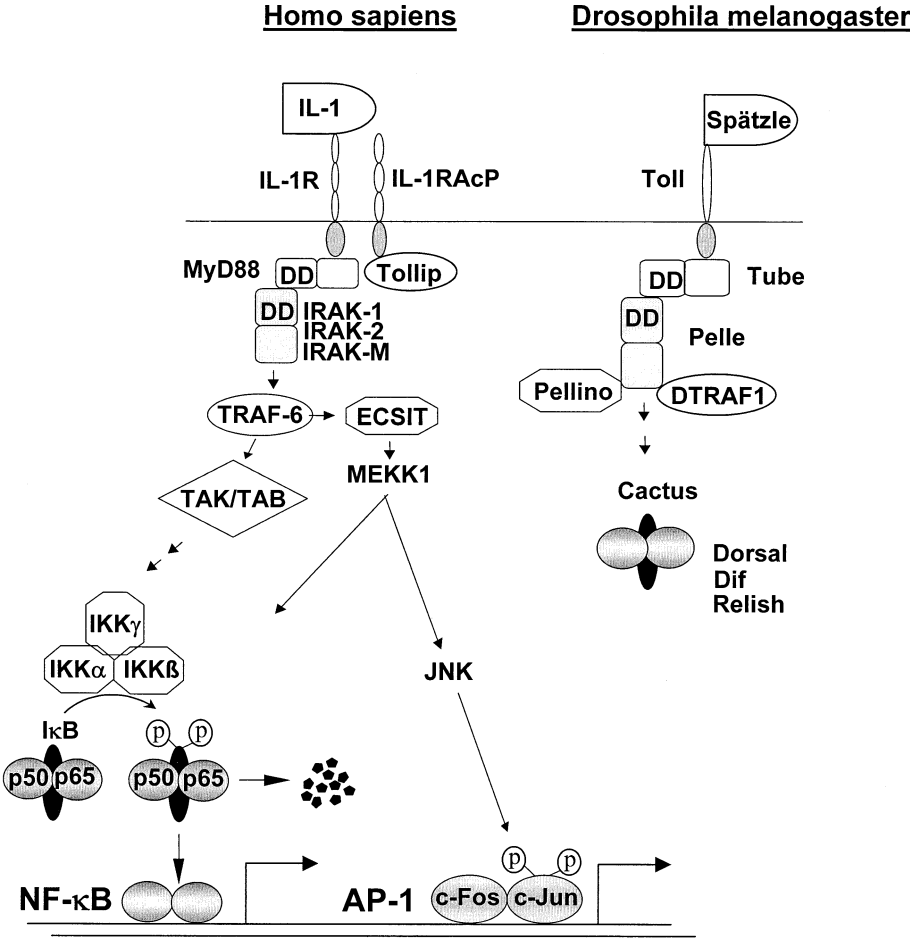

Fig. 1. Schematic comparison of the signaling pathways elicited by IL-1 in *Homo sapiens* (left) and Spätzle in *Drosophila* (right). Proteins with sequence similarities are shown by similar shading. For further details, see text.

2. Materials and methods

2.1. Expression vectors, reporter plasmids and antibodies

The reporter plasmids 3x(κB)-luc and 3xAP-1-luc [16] and the expression vectors encoding HA-tagged JNK1 and JNK2 [17] were described. The eukaryotic expression vectors for the *Drosophila* proteins were constructed as follows: pcDNA-3 Tube was cloned by inserting the Tube cDNA as an *Eco*RI/*Xho*I fragment into pcDNA-3, Pelle was cloned as an *Eco*RI/*Xba*I fragment into pcDNA-3. The Pelle K240R point mutant was generated using the Quickchange Kit (Stratagene) according to the instructions of the manufacturer. PelleΔC was generated by removing the *Sac*II/*Xba*I fragment from pcDNA-3 Pelle. The Torso fusion protein expression vectors were cloned as described [13]. Details about the polymerase chain reaction primers and the generation of the plasmid constructs can be obtained from the authors upon request. Antibodies recognising the HA epitope (12CA5) were purchased from Roche Molecular Biochemicals, α-phospho-p38 and α-phospho-ERK1/2 antibodies were from New England Biolabs.

2.2. JNK assays

Cells were lysed in NP-40 lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 0.5 mM sodium vanadate, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1% (v/v) NP-40 and 10% (v/v) glycerol) and the JNK proteins were immunoprecipitated by the addition of 1 μg of αHA antibodies and 25 μl of protein A/G plus agarose. The precipitate was washed three times in lysis buffer and twice in kinase buffer (20 mM HEPES/KOH pH 7.4, 2 mM dithiothreitol (DTT), 25 mM β-glycerophosphate, 20 mM $MgCl_2$). The kinase assay was performed in a final volume of 20 μl kinase buffer containing 5 μCi [γ-^{32}P]ATP, 20 μM ATP and 2 μg of bacterially expressed GST-c-Jun protein. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 5×sodium dodecyl sulphate (SDS) loading buffer and separated by SDS–PAGE. The gel was fixed, dried and exposed to an X-ray film.

2.3. Luciferase assays

Cells were washed with phosphate-buffered saline and lysed in reporter lysis buffer (25 mM Tris–phosphate, 2 mM DTT, 2 mM CDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100). Luciferase activity was measured in a luminometer (Duo Lumat LB 9507, Berthold) by injecting 50 μl of assay buffer (40 mM tricine, 2.14 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 5.34 mM $MgSO_4$, 0.2 mM EDTA, 66.6 mM DTT, 540 μM CoA, 940 μM luciferin, 1.06 mM ATP), followed by the measurement of light emission for 10 s.

2.4. Cell culture and transient transfections

Human HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. For analysis of luciferase activity, approximately 1×10^5 HeLa cells were grown in six-well plates and transfected using the Superfect® reagent (Qiagen Inc.) according to the instructions of the manufacturer. The amount of transfected DNA (2.5 μg) was kept constant with empty expression vector. JNK activation was measured by transfecting 1×10^6 HeLa cells with 0.25 μg of HA-tagged JNK1 and JNK2 along with 2 μg of expression vector encoding the various *Drosophila* proteins, respectively.

2.5. Western blot analysis

Cell extracts contained in NP-40 lysis buffer were separated on a 12% reducing SDS–polyacrylamide gel. Subsequently the proteins were transferred from the SDS gel onto a polyvinylidene difluoride membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad). Prior to the incubation with the primary antibodies, the membrane was blocked with 5% non-fat dry milk powder in TBST buffer (25

mM Tris–HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.05% (v/v) Tween-20). The primary antibodies were detected with appropriate secondary antibodies coupled to horseradish peroxidase and visualised by enhanced chemiluminescence according to the instructions of the manufacturer (NEN Lifescience).

3. Results and discussion

In order to functionally analyse the role of the *Drosophila* *melanogaster* proteins Pelle and Tube in the vertebrate IL-1 signaling system, both cDNAs were cloned into a mammalian expression vector. We also constructed expression plasmids for a Pelle protein lacking its kinase function (Pelle K240R) due to a point mutation within the ATP binding loop [18] and a Pelle protein lacking its C-terminus (PelleΔC). Since the oligomerisation of Pelle and Tube is important for its biological function in *Drosophila* [13], both proteins were also fused to the extracellular part and membrane-spanning region of a gain-of-function allele of the receptor tyrosine kinase Torso. The Torso portion presumably directs plasma localisation and, due to a tyrosine to cysteine exchange at position 327, ligand-independent oligomerisation of the fusion protein [19]. The eukaryotic expression vectors used in this study are schematically displayed in Fig. 2A.

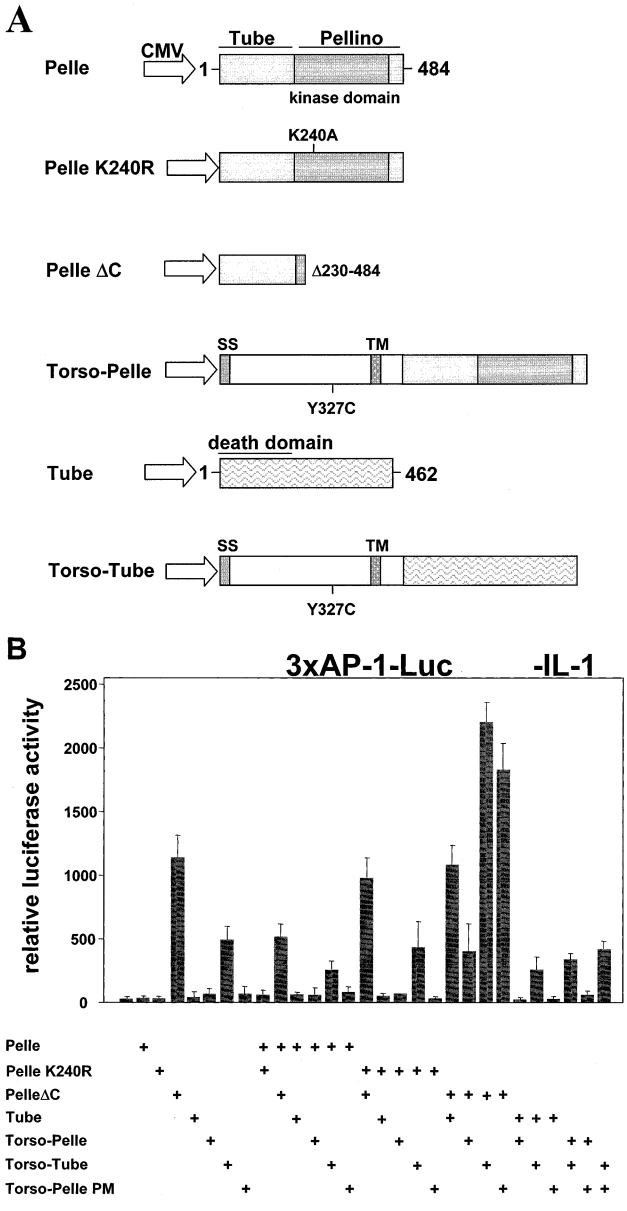

Fig. 2. Activation of AP-1 upon expression of Torso–Tube and PelleΔC. A: Structure of Tube, Pelle and the Torso fusion proteins. The interaction domains of Pelle with Tube and Pellino are indicated, the N-terminal DD of Tube is shown. The indicated subregions within Torso are the signal sequence (SS), the splitted tyrosine kinase domain (Y327C) and the transmembrane (TM) region. The arrow shows the cytomegalovirus (CMV) promoter, which allows expression of proteins in eukaryotic cells. B: HeLa cells were transiently transfected with an AP-1-dependent reporter gene together with the indicated expression vectors (1 μg) or with empty expression vector as a control. 2 days later, luciferase activity was determined and gene expression was measured as relative luciferase activity. Mean values from at least four independent experiments are shown, standard deviations are indicated by bars.

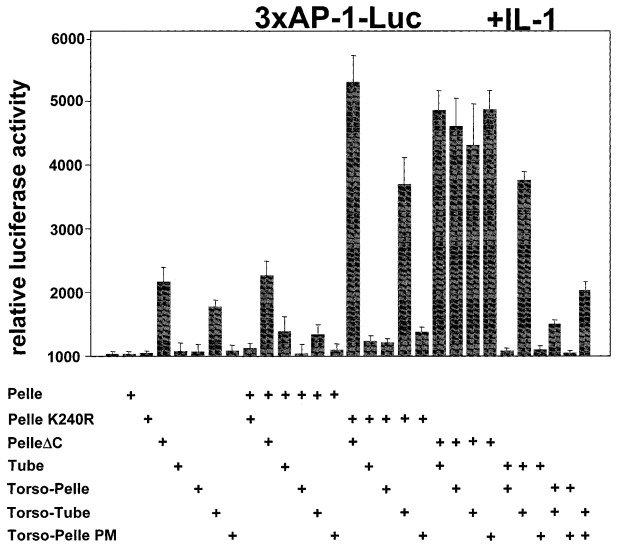

Fig. 3. Activation of AP-1-dependent gene expression by Torso–Tube and PelleΔC in the presence of IL-1. HeLa cells were transfected with the AP-1 reporter gene and the indicated Pelle/Tube expression vectors as shown. 36 h later, cells were stimulated for 12 h with IL-1β (1 ng/ml). Luciferase activity was determined and displayed as in Fig. 2B, bars show standard deviations from at least four experiments.

To test a possible function of these *Drosophila* proteins for the activation of NF-κB in a vertebrate system, HeLa cells were cotransfected with a NF-κB-dependent luciferase reporter gene and all possible combinations of expression vectors encoding wild-type and mutant forms of Pelle or Tube. None of the combinations was able to efficiently activate the NF-κB-dependent reporter gene, although Western blot experiments ensured that the *Drosophila* proteins were properly expressed (data not shown). The fly proteins also failed to induce DNA binding of NF-κB in various cell lines (data not shown), indicating that the structural similarity of the Toll/Dorsal signaling cascade to the mammalian NF-κB system is not conserved at the functional level. The lack of Pelle/Tube function for NF-κB activation may be attributed to the differences in the architecture of Pelle/Tube and MyD88/Pelle complexes. While the IRAK/MyD88 interaction occurs via DDs contained in both proteins, binding of Pelle/Tube is mediated by sequences outside of the DD which are not conserved in either MyD88 or IRAK [20].

Since IL-1 also activates AP-1, we then tested the effects of the *Drosophila* proteins on the activity of a luciferase reporter

gene under the control of three AP-1 binding sites (Fig. 2B). Although Pelle and Tube failed to induce AP-1 activity either alone or in combination, the expression of PelleΔC stimulated the AP-1 reporter gene. This stimulatory effect occurred upon expression of PelleΔC either alone or in combination with any other of the various Pelle or Tube protein variants. This unexpected behaviour shows that the AP-1-inducing function of Pelle is not dependent on its kinase function. The gain of Pelle function upon removal of its C-terminal part may indicate that the C-terminus of Pelle is bound by an inhibitory protein, possibly a homologue to the *Drosophila* protein Pellino. Alternatively, these results would be compatible with a model in which removal of the Pelle C-terminus allows the adoption of its active conformation. AP-1 activity is also induced by Torso–Tube, the strongest expression of the reporter gene was seen upon coexpression of PelleΔC with Torso–Tube. Since Torso–Pelle fails to induce AP-1 activity it is reasonable to assume that Torso–Tube-mediated transcription is mediated by Tube rather than by the Torso protein. These results show that a membrane-bound and multimerised form of the *Drosophila* protein Tube functions in the activation of mammalian AP-1. This result is in accordance with a previous study showing that oligomerisation is required for the full functionality of Tube in *Drosophila* development, as revealed by genetic experiments [13]. It will be interesting to learn, whether the functionality of Tube for AP-1 activation requires membrane recruitment, multimerisation, or both.

We then tested the AP-1-inducing capacity of Pelle and Tube proteins in the presence of IL-1. HeLa cells were transfected with an AP-1-dependent reporter gene along with all combinations of expression vectors encoding the *Drosophila* proteins and then stimulated with IL-1 (Fig. 3). IL-1-induced reporter gene activity was further enhanced only upon expression of PelleΔC or Torso–Tube, albeit to quite different extents. While the expression of Pelle together with Tube failed to activate AP-1-dependent gene expression in the absence of IL-1, this combination slightly activated the AP-1-dependent reporter gene in the presence of this cytokine.

Since AP-1-dependent gene expression is enhanced by JNK-mediated phosphorylation of the AP-1 family members c-Jun, JunD and JunB [21], we determined the effects of Pelle or Tube expression on the activity of this MAPK. HeLa cells were transfected with vectors encoding various combinations of Pelle and Tube proteins together with HA-tagged JNK1 and JNK2. Tagged JNK proteins were immunoprecipitated and their activity was determined by measuring phosphoryla-

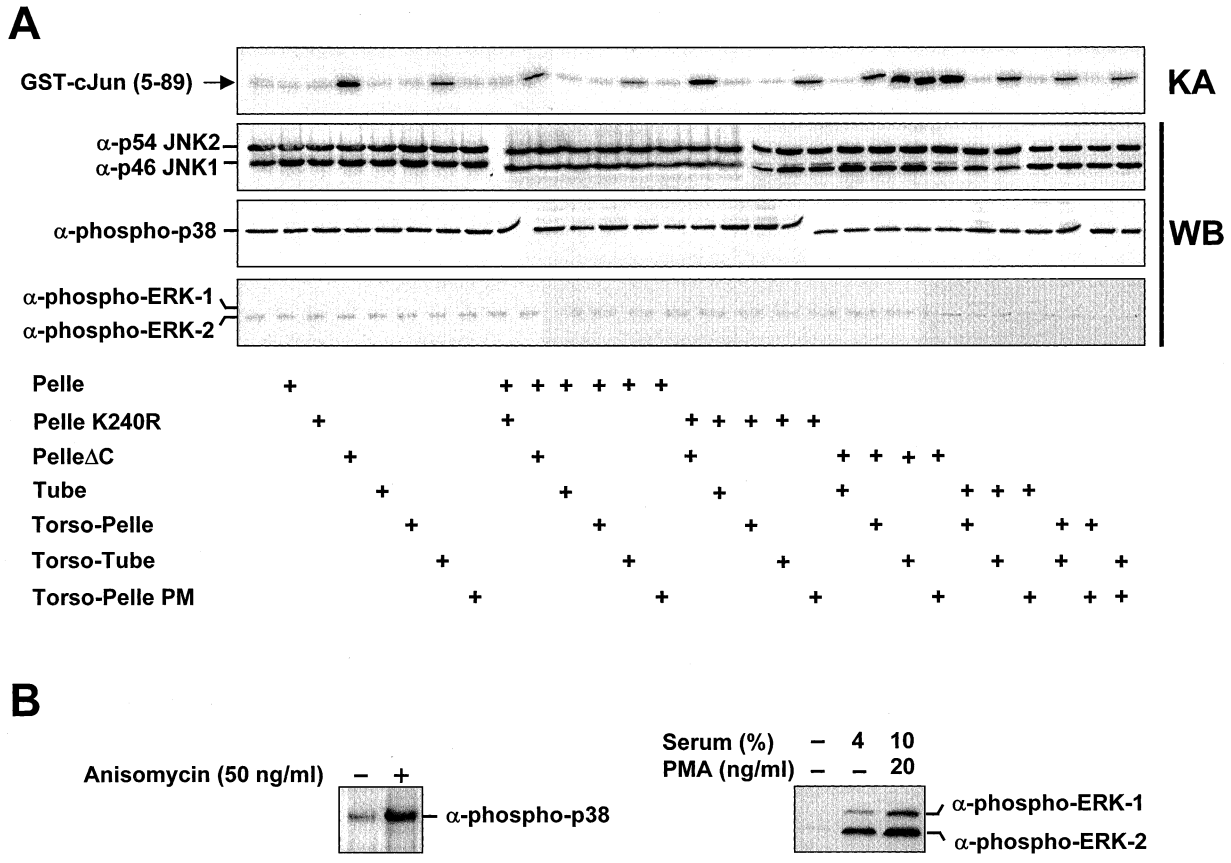

Fig. 4. Analysis of Torso–Tube and PelleΔC-induced MAPK activity. A: HeLa cells were transfected as indicated with HA-tagged JNK1 and JNK2 expression vectors along with the plasmids encoding the various *Drosophila* proteins or with empty expression vector as a control and plated on two dishes. 2 days later, cells grown on the first dish were lysed and aliquots thereof analysed for multiple parameters. One fraction of the extracts was tested for JNK activity by immunoprecipitation of HA-tagged JNK1/2 from cell lysates, followed by determination of kinase activity by immune complex kinase assays (KA) using recombinant GST-c-Jun (5-89) as substrate. An autoradiogram from reducing SDS gels is shown. Further fractions of the total extracts were analysed by Western blotting (WB) for HA-tagged JNK1 and JNK2 or for phosphorylated and activated forms of endogenous p38. Transfected cells plated on the second dish were grown for 2 days in medium containing 0.5% FCS, lysed and tested by immunoblotting for the occurrence of phosphorylated ERK1/2 (lower). B: HeLa cells were stimulated for 10 min as indicated and subsequently analysed for the induced phosphorylation of endogenous p38 and ERK1/2. Representative Western blots and autoradiograms from reducing SDS gels are displayed.

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Drosophila melanogaster MAYGWNGCGMGVQVNGSNGAIGLSSKYSRNTELRRVEDNDIYRLAKILDENSCWRKLMSI 60
Drosophila virilis      MEMAKANGWAVVCTSTNTNTVPIYSKYTRCTELRRVDDNDIYKLATILDVNGCWRKLMSI 60
Bombyx mori             ---------------------------ERNTELRKLPMGSLYNIINILEINDSWQKVMAW 33
                                                     * ****::  ..:*.: .**: *..*:*:*:

                        IPKGMDVQACSGAGCLNFP-AEIKKGFKYTAQDVFQIDEAANRLPPDQSKSQMMIDEWKT 119
                        IPKRLDAQACSAPGALNYQEIAAKVGLKYTAQQISLIDGTAERLTPGQSISQVMIDEWKT 120
                        IPTNPQSDHFHR---------------KYNSEHLRMIQDDAK--ISKRTCSEILFDEWST 76
                        **.  : :                   **.::.:  *:  *:   . :: *::::***.*

                        SGKLNERPTVGVLLQLLVQAELFSAADFVALDFLNESTPARPVDGPGALISLELLEEEME 179
                        SGKLNERPTVGVLLQLLVHAEIYSAADFVALHFLNEPKPERPTDGPAAHISLDLCSEDLS 180
                        SGRIR--PTVATLLDVLVKAEIYRAADEIAN-ILGEPLPPRPLEGPAAKIDTN------- 126
                        **::.  ***..**::**:**:: *** :*  :*.*. * ** :**.* *. :

                        VDNEGLS-LKYQSSTATLGADAQGS--VGLNLDNFEKDIVRRDKSVPQPSGN----TPPI 232
                        EDMDVEDGASYQPNTSALNAAVEQARGTGMNLDYFDKHMVRRDKSVPQQLENGTSSTVPV 240
                        ------------VTFMLNGESMPLDGSDVNLKDNQNLDTKNTNQHP------------- 160
                                     *  *..         .:**.  ::  .:. :. *

                        APPRRQQRS----TTNSNFATLTGTGTTSTT-IPNVPNLTILNPSEQ----IQEPVLQPR 283
                        PPPRAARSSRLLKATASNVAPTTASNAPSASNTANVPNLSILNASSKKLAASSEQTLQPQ 300
                        ----------------DLHNRTTKQLKSADPLIIFSNDNISNGPKD------------- 190
                                        :.   * .   *:     ..* .* * ...

                        PMNIPDLSILISNS---------GDLRATVSDNPSN-----RTSSTDPPNIPRITLLIDN 329
                        --NIPNLSILNGSSEAVLMATTSTTLDAGKSDNASNGRSSASTSTATIPNVPLITLLIEN 358
                        ----------------------------------------SKSFGHKTTAQVISYERN 208
                                                               :.:    . . :     *

                        S-------GDVNSRPNHAPAKASTATTPTASSNNLPMISALNISKGS------------K 370
                        SSCEISDASDATQITSKSTATKTVPDMSTASYNNLPAISALNLNIASGAGELDGNGAKAR 418
                        S----------------------AYRSTLRTEELPNISALMG----------------- 228
                        *                      .  .*   ::** ****
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Fig. 5. Sequence alignment of Tube proteins. The sequence from the *D. melanogaster* Tube protein was aligned with its homologues using the EMBL Clustalw program, numbers corresponding to the residues in each sequence are shown on the right. The GenBank accession numbers are: *D. melanogaster*: P22812, *D. virilis*: Q08171, *B. mori*: AU000062. Identical amino acids are shown in bold and marked by stars, homologous and related amino acids are indicated.

tion of the exogenously added substrate protein GST-c-Jun (5-89) in immune complex kinase assays (Fig. 4A). As already seen for the activation of AP-1, only expression of PelleΔC and Torso–Tube resulted in the induction of JNK activity. In parallel, cell extracts were also analysed for the activity of two other members of the MAPK family, namely p38 and extracellular signal-regulated kinase (ERK). Determination of p38 and ERK1/2 activation by immunoblotting using phospho-specific antibodies revealed that the *Drosophila* proteins did not affect activation of p38 (as measured by p38 phosphorylation at Thr180/Tyr182) or ERK1/2 (as determined by phosphorylation at Thr202/Tyr204). In control experiments, phosphorylation of both MAPKs was augmented by known activators (Fig. 4B). In summary, these experiments show that *Drosophila* proteins PelleΔC and Torso–Tube selectively activate the JNK pathway in mammalian cells. In contrast, Pelle and Tube do not affect the ERK1/2, p38 or NF-κB pathways. This might indicate that the *Drosophila* proteins induce the JNK cascade at a position after bifurcation of the JNK and NF-κB signaling pathways which might take place at the level of TRAF-6.

Similar to the Toll/Dorsal proteins, also the *Drosophila* JNK protein (DJNK) is involved in embryonic pattern formation and the immune response. Furthermore, the JNK signaling pathway is highly conserved between mammals and flies [21], making it plausible that *Drosophila* proteins function in this conserved pathway in mammalian cells. The functionality of *Drosophila* proteins in human cells is not without precedent. The *Drosophila* TRAF-1 (DTRAF-1) protein interacts not only with Pelle, but also with human TRAF-1, TRAF-2, TRAF-4 and IAP (inhibitor of apoptosis) proteins [22]. In accordance with our results, this study also revealed a lack of NF-κB activation upon expression of Pelle alone [22]. The same study showed that expression of Pelle together with DTRAF-1 weakly induced NF-κB activity.

The functionality of Tube in HeLa cells raises the question for a mammalian Tube homologue. Computer database searches identified two Tube-related proteins in insects (Fig. 5), but did not reveal any indication of Tube homologues in mammals. We also failed to isolate a Tube homologous cDNA when screening an *Amphioxus* cDNA library at low stringency (data not shown). As described, the *D. melanogaster* Tube protein has a structural and functional homologue in *Drosophila virilis*. These two *Drosophila* Tube proteins share sequence similarity to a protein sequence predicted from a partial *Bombyx mori* cDNA. Alignment of the three sequences showed already a remarkable degree of sequence diversity within the insect kingdom, making the existence of Tube homologue in mammals rather unlikely. The regions shared by the three Tube proteins corresponds to the DD, a family of folds identified in proteins that assemble components of apoptotic signaling pathways [4].

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