REVIEW

INFLUENZA A VIRUS PB1-F2 PROTEIN

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Summary. – PB1-F2 protein (PB1-F2) is encoded by the alternative (+1) ORF in the PB1 gene of influenza A viruses (IAVs). This protein has a number of unique features, namely its absence from some animal IAV isolates, variable expression in individual infected cells, rapid proteasome-dependent degradation, mitochondrial localization, and apoptotic or pro-apoptotic properties. Localization of PB1-F2 to mitochondria is mediated via C-terminal basic amphipathic α-helix. PB1-F2 affects apoptosis and may contribute to the pathogenicity and lethality of IAVs. Sequence analysis showed that, in addition to the strains with an ORF for full-length PB1-F2, there are some with an ORF for different truncated forms of PB1-F2. Several other viruses encode proteins with structure and function similar to PB1-F2 of IAVs.

Key words: Influenza A virus; PB1-F2; ORF; mitochondrial membrane amphipathic α-helix; apoptosis; truncation

Contents:

1. Introduction

Influenza is a viral disease of global dimension associated with high morbidity and mortality in annual epidemics and infrequent pandemics with very high attack rates (Hilleman, 2002). The most extreme pandemic in the 20th century was Spanish influenza in 1918–1919, which killed an estimated 20–40 million people throughout the world (Potter, 2001; Taubenberger *et al*., 2001).

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Abbreviations: ANT3 = adenine nucleotide translocator 3; HA = hemagglutinin; IAV(s) = Influenza A virus(es); M1 = matrix protein; M2 = ion channel protein; MMP = mitochondrial membrane permeabilization; NA = neuraminidase; NP = nucleoprotein; PB1–F2 = PB1–F2 protein; PR8 = A/Puerto Rico/8/34 (H1N1) strain; PTPC = permeability transition pore complex; VDAC1 = voltage-dependent anion channel 1

IAVs have a negative-strand RNA genome which consists of eight RNA segments encoding eleven proteins: two surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA), matrix protein (M1), ion channel protein (M2), nucleoprotein (NP), three proteins of polymerase complex – PB1, PB2 and PA, and two non-structural proteins – NS1 and NS2 (Steinhauer and Skehel, 2002). IAVs are classified into serologically defined antigenic subtypes of HA and NA (HN subtypes). Sixteen H and nine N subtypes of IAVs have been so far serologically identified (Kaye and Pringle, 2005). Since the beginning of the 20th century, only the H1, H2, and H3 subtypes of HA and the N1 and N2 subtypes of NA have been associated with stable infection in humans resulting in recurrent annual epidemics (Lewis, 2006)

While searching for IAVs alternative ORF proteins that are recognized by CD8+T cells, PB1-F2 encoded by the (+1) ORF in the PB1 gene was discovered (Chen *et al*., 2001). In addition to its mode of translation, PB1-F2 has several unique features. These include its absence from some animal IAV isolates, variable expression in individual infected cells, rapid proteasome-dependent degradation, mitochondrial localization, and apoptotic or pro-apoptotic properties. The exposure of cells to a synthetic version of PB1-F2 induced apoptosis and resulted in variably sized pores in planar lipid membranes (Chanturiya *et al*., 2004). PB1-F2 sensitized cells to apoptotic stimuli and uniquely interacted with both the inner and outer mitochondrial membranes (Zamarin *et al*., 2005).

Furthermore, recent studies also confirmed the role of PB1-F2 in pathogenesis. IAVs knocked out for the expression of PB1-F2 and its downstream initiation products were not attenuated in replication in tissue culture, but their pathogenicity and lethality for mice was considerably reduced. Also, the PB1-F2-knockout viruses were cleared from the lungs more rapidly and induced earlier immune response to the infection, implying that PB1-F2 played a role in suppressing an immune response responsible for viral clearance (Zamarin *et al*., 2006).

2. Primary structure of PB1-F2

PB1-F2 was discovered in the A/Puerto Rico/8/34 (H1N1) strain (PR8). PR8 encodes the relatively small 87-aa PB1-F2. This protein is translated from an alternative (+1) ORF in the polymerase PB1 gene (nt 120–381). In the majority of IAVs, this ORF codes for 90-aa PB1-F2 (by 3 aa longer than the PB1-F2 of PR8). These extra three amino acids in the 90-aa PB1-F2 are to a certain extent conservative (Pančuchárová and Russ, 2006). Some IAVs have an ORF for truncated PB1-F2 of various length due to the presence of premature stop codons in the sequence. The incidence of an ORF for truncated PB1-F2 is discussed below.

2.1. Surprising variability of PB1-F2

PB1-F2 is encoded by the alternative ORF (+1) in the highly conservative polymerase PB1 gene. In analyzing the sequences of putative PB1-F2 proteins in human IAVs we found that 18 aa (at the positions 1, 5, 7, 8, 9 12, 13, 15, 19, 20, 24, 61, 64, 72, 77, 85, 88, and 89) are conservative in human isolates, while only 5 aa are absolutely conservative in the isolates from all hosts (Table 1, Fig. 1) (Pančuchárová and Russ, 2006). This rather surprising outcome of sequence analysis is in a good agreement with the results of Obenauer *et al*. (2006) who showed that PB1-F2 is, paradoxically, the

Table 1. Comparison of the variability of each genome segment expressed as a percentage of total (variability) and the number of amino acid variations per position in avian vs. non-avian hosts (modified according to Obenauer *et al*., 2006)

HA	No. of analyzed IAVs	No. of conservative amino acids	% of conservative amino acids IAVs
H1	48	31/90	35%
H2	24	63/90	70%
H3	51	53/90	59%
H5	22	62/90	69%
Σ	148	15/90	17%

Fig. 1

Distribution of conservative amino acids in PB1-F2 in human (A) and all analyzed isolates of IAV (B)

third most variable protein besides HA and NA in avian IAVs and the sixth most variable one in non-avian IAVs (Table 2).

Table 2. Incidence of conservative amino acids in PB1-F2 of human IAVs (Pančuchárová and Russ, 2006)

Genome segment	Variability (%)	Variants/Position	
		Avian hosts	Non-avian hosts
PB1	4	1.52	1.52
PB1-F2		3.54	2.45
PB2	4	1.67	1.66
PA	4	1.74	1.70
HA	32	5.52	4.42
NP	4	1.81	1.85
NA	31	5.09	4.38
M	4	1.84	1.77
M2	5	3.50	3.42
NS	7	3.40	2.89
NS2	5	2.81	2.68

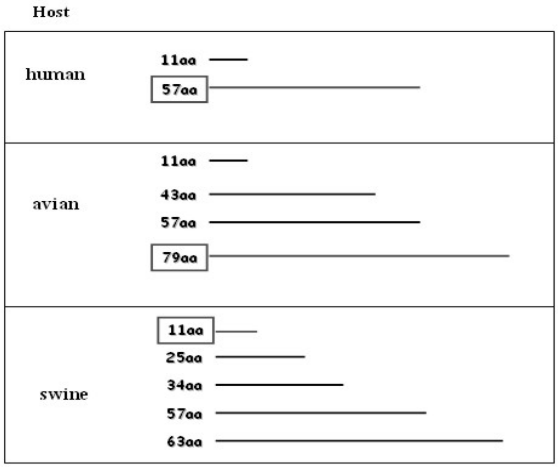

Fig. 2

Length of the ORFs encoding PB1-F2 in human, avian and swine isolates of IAV

Prevalent forms are marked.

2.2. Incidence of ORF for truncated PB1-F2

The analysis of the PB1 gene of 42 IAV isolates from Taiwan, including 24 H1N1 and 18 H3N2 isolates**,** revealed that most of the H1N1 isolates contained an ORF for a putative shorter 57-aa PB1-F2 encountering a premature stop codon (Chen *et al*., 2004). However, all of the Taiwanese H3N2 isolates contained an ORF for full-length PB1-F2 except for A/Taiwan/1748/97, which might encode a C-terminally truncated PB1-F2 of 79 aa.

In analysing the variability of PB1-F2 we found that the length of ORF was different and depended on the type of host (Fig. 2), HA subtype, and sometimes also the year of virus isolation (Pančuchárová and Russ, 2006). We found that 67% of human H1 isolates, 47% of swine H1 isolates, and 48% of avian H9 isolates had an ORF for truncated PB1-F2 (Table 3). Interestingly, the H1N1 viruses are also known to cause a disease less frequently than H3N2 viruses. In summary, 16% of all analyzed IAV isolates might encode a putative C-terminally truncated PB1-F2. A similar proportion, 13% of truncated PB1-F2, was described by Zell *et al*. (2007). Zamarin *et al.* (2006) showed that, in addition to full-length protein, the C-terminal region of PB1-F2 could be also simultaneously expressed from the downstream second initiation codon to produce a N-terminally truncated protein (Fig. 3). We found that this second AUG of the PB1 gene region is not completely conservative (Pančuchárová and Russ, 2006).

Table 3. Incidence of IAVs of human, avian and swine origin with truncated PB1-F2 (Pančuchárová and Russ, 2006)

Host		No. of analyzed IAVs	% of IAVs with truncated PB1-F2
Human	H1	48	67%
	H2	24	0%
	H3	51	0%
	H5	22	0%
	H9	3	0%
	Σ	148	22%
Avian	H1	3	33%
	H2	12	0%
	H3	37	5%
	H4	19	0%
	H5	156	1%
	H6	29	0%
	H7	18	0%
	H8	1	0%
	H9	101	48%
	H10	11	0%
	H11	5	0%
	H12	5	0%
	H13	1	0%
	H14	1	0%
	H15	3	0%
	H16	1	0%
	Σ	403	13%
Swine	H1	34	47%
	H2	34	3%
	H3	1	0%
	H5	2	0%
	H9	4	0%
	Σ	75	23%
Total		626	16%

Fig. 3

Nucleotide sequence of PB1-F2 gene of A/Wisconsin/3523/88 (H1N1)

Positions of two start codons are shown.

3. Secondary structure of PB1-F2

The PB1-F2 molecule can be divided into two approximately equally sized domains. These correspond to a N-terminal domain, for which little secondary structure was predicted, and a C-terminal domain that exhibits a considerable tendency for α-helical structure (Fig. 4) (Henklein *et al*., 2005). Furthermore, there is a relatively high number of positively charged amino acids with side chains and tryptophans located within the C-terminal helix region. Within the 44 aa of the C-terminal region a total of 10 positive net charges are found, with a clustering of 6 aa in the region 73–85 and 3 tryptophans at the positions 58, 61, and 80, of which Trp61 is fully conserved (Gibbs *et al*., 2003). The high occurrence of cationic amino acids together with the high tendency for α-helix formation predicts a model of the complete molecule that has amphipathic character (Gibbs *et al.*, 2003; Yamada *et al.*, 2004). For the region 65–85, which contains 35% of lysines and arginines a helical structure is predicted. Accordingly, this part of PB1-F2 resembles cationic antimicrobial peptides (Hancock and Lehrer, 1998).

4. Mitochondrial localization of PB1-F2

PB1-F2 and PB2 are the only two proteins of IAVs that may localize in the mitochondria of host cells (Chen *et al.*, 2001; Carr *et al*., 2005). In infected cells, 55% of PB1-F2 was detected in mitochondria and the rest was found in nucleus and cytosol (Gibbs *et al*., 2003). PB1-F2 localization in mitochondrial membrane is mediated via C-terminal basic amphipatic α-helix. PB2 interacts with mitochondria by its N-terminal region with predicted α-helical secondary structure containing 22 aa (Woodfin and Kazim, 1993).

There is a number of viral proteins that have similar mitochondrial localization; e.g. Vpr protein of Human immunodeficiency virus 1, K7 protein of Kaposi's sarcoma-associated herpesvirus), $p13^{II}$ protein of Human T-cell

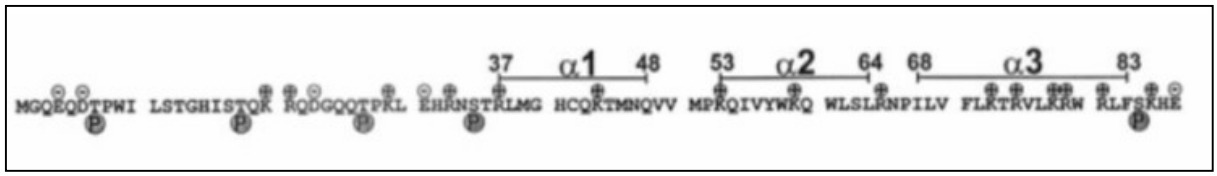

Fig. 4

The amino acid sequence of PB1-F2 of IAV PR8

Predicted secondary structures, phosphorylation sites, and positions of positively and negatively charged side chains (Henklein *et al*., 2005).

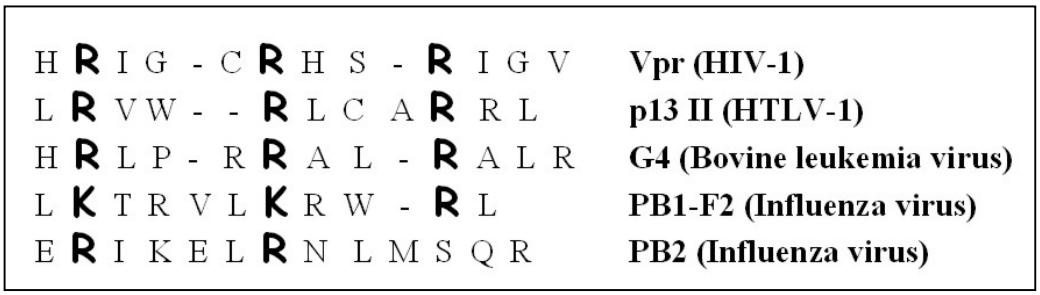

Fig. 5

Alignment of α-helical domains of mitochondrial-targeted proteins of five different viruses (modified according to Boya *et al*., 2003)

leukemia virus-1, HVB-X of Hepatitis B virus, and M11L protein of Human cytomegalovirus. Some of these proteins possess α-helical and arginine-rich mitochondriotoxic domains (Fig. 5). All these proteins induce morphological changes in mitochondria and most of them have apart from mitochondrial also nuclear localization.

Exposure of cells to a synthetic PB1-F2 induced morphological changes in mitochondria, including changes of cell shape, partial fragmentation and loss of membrane potential. Synthetic PB1-F2 in nanomolar concentration induced potential-dependent increase in trans-membrane conductivity. Thus PB1-F2 decreased the stability of membranes via generating potential-dependent lipid pores that led to disruption of their structure. The increase in conductivity was most evident with monovalent cations, chloride anions and, to a small extent, also bivalent ions. Similarly, Vpr protein of HIV-1 also formed cation-selective ion channels in planar lipid bilayers (Piller *et al*., 1999).

Studies with synthetic PB1-F2 revealed the ability of its C-terminal part to associate with cellular membranes and to lyse cells, bacteria and red blood cells with similar potency as antimicrobial agents melittin or mastoparan.

According to the data mentioned above it might be suggested that the cell death induced by synthetic PB1-F2 is caused by permeabilization and destabilization of mitochondrial membrane resulting in the leakage of macromolecules and subsequent cell death (Chen *et al*., 2001).

5. Role of PB1-F2 in apoptosis

Apoptosis is an active death program that contributes to the elimination of damaged, mutated, aged, or virus-infected cells (Ferri and Kroemer, 2001). Apoptosis may be initiated by an extrinsic pathway, in which death receptors expressed at the cell surface trigger the receptor-proximal activation of caspases and, subsequently, mitochondrial membrane permeabilization (MMP). When the cell death is triggered by an intrinsic pathway, death signals act directly on mitochondria leading to MMP before caspases are activated. The MMP is a critical step in the regulation of apoptosis, namely it leads to the release of pro-apoptotic factors, some of which can activate caspases (a family of proteins that serve as cellular demolition experts), while others can activate caspase-independent death pathways (Boya *et al*., 2004).

Viruses have evolved multiple strategies to modulate apoptosis for their own benefit. Thus many viruses code for proteins, both anti-apoptotic and pro-apoptotic, which act on mitochondria and control apoptosis of infected cells. Viral pro-apoptotic proteins translocate to mitochondrial membranes and induce MMP that is often accompanied by mitochondrial swelling and fragmentation (Boya *et al*., 2004). Frequently, mitochondrial target signal consists of a region of 20–60 aa with abundant positive charges and hydroxylated residues forming amphipathic α-helices in membranes (Neupert, 1997). This structure is necessary for pro-apoptotic effects and seems to have pore-forming properties, as it has been shown for Vpr protein of HIV-1 (Schuler *et al*., 1999) and PB1-F2 of IAV (Chen *et al*., 2001). Influenza virus infection results in the activation of cellular pathways aimed at inhibition of viral replication and induction of an antiviral state (Garcia-Sastre, 2001). To overcome the antiviral signaling, influenza viruses evolved accessory proteins, such as NS1 and PB1-F2, which have been proposed to down-modulate different aspects of the host immune response.

In characterizing the mechanism of action for PB1-F2, Zamarin *et al.* (2005) have observed a sensitization of cells to apoptotic stimuli (e.g. tumor necrosis factor alpha) by PB1-F2. Moreover, a treatment of purified mouse liver mitochondria with recombinant PB1-F2 resulted in cytochrome c release, loss of the mitochondrial membrane

Table 4. Mitochondrion – a target of viral anti-apoptotic and pro-apoptotic proteins. (modified according to Boya *et al.*, 2003)

Class of modulators	Viral protein	Putative molecular target
Anti-apoptotic	v MIA (Human cytomegalovirus)	ANT
	M11L (Myxoma virus)	PBR
	K7 (Kaposi's sarcoma-associated herpesvirus	Bcl-2, caspase-3, CAML
Pro-apoptotic	Vpr (HIV-1)	ANT
	$p13^{II}$ (HTLV-1)	?
	PB1-F2 (Influenza A virus)	ANT, VDAC
	HVB-X (Hepatitis B virus)	VDAC

potential, and enhancement of tBid-induced mitochondrial permeabilization, suggesting a possible mechanism for the observed cellular sensitization to apoptosis. Mitochondrial targets for PB1-F2 have been identified. PB1-F2 uniquely interacts with the inner mitochondrial membrane adenine nucleotide translocator 3 (ANT3) and the outer mitochondrial membrane through a voltage-dependent anion channel 1 (VDAC1), both of which form a permeability transition pore complex (PTPC) during apoptosis. The interaction with PTPC proteins is typical for a number of viral apoptotic proteins (Table 4).

On the basis of these findings, a model was proposed. According this model the pro-apoptotic PB1-F2 acts through mitochondrial PTPC and may play a role in the down-regulation of the host immune response to infection (Fig. 6). The PB1-F2-induced mitochondrial permeabilization can proceed through three possible mechanisms: (i) enhancement of the pore complex formation through direct interaction with ANT3 and VDAC1, (ii) independent permeabilization of the inner and outer mitochondrial membranes with the help of ANT3 and VDAC1, respectively, and (iii) direct permeabilization of mitochondrial membranes. It is possible that PB2 may also influence the outcome of the apoptotic response via its mitochondrial targeting. In fact, Morris *et al.* (2005) have recently suggested a role for PB2 in determining the apoptosis in infected cells. Namely, PB2 would act antagonistically to PB1-F2. PB2 might counteract the negative mitochondrial

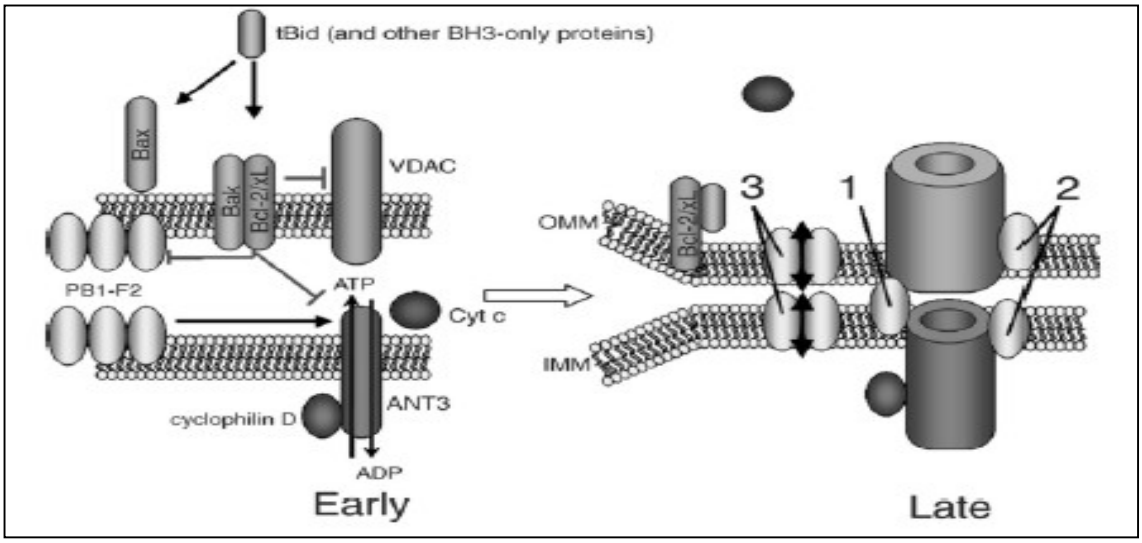

Fig. 6

Model of action of the PB1-F2 during infection

During early stages of the infection, PB1-F2 localizes to mitochondria, where it interacts with ANT3 and VDAC and predisposes the mitochondria to permeability transition. Later in the infection, when more PB1-F2 is synthesized, and, upon induction of antiviral apoptotic signaling pathways, the mitochondria undergo the permeability transition, which results in the induction of apoptosis (Zamarin *et al.,* 2005).

effect of PB1-F2 in infected cells. However, it is important to note that several influenza viral proteins have been proposed to influence the apoptotic response. Indeed, the apoptosis induced by IAVs appears to be a multi-factorial process.

6. Role of PB1-F2 in viral pathogenesis

PB1-F2 had no substantial effect of on viral gene expression or virus-induced apoptosis in epithelial cell lines supporting productive influenza virus infection (Chen *et al*., 2004). Furthermore, while PB1-F2 was shown to be more apoptotic in immune cells, implying its possible role in modulation of immune response, the significance of this finding was never demonstrated within the context of infection of an animal host. Zamarin *et al.* (2006) have attempted to clarify the role of PB1-F2 in viral infection by determining its involvement in viral pathogenicity for mice.

Interestingly, Chen *et al*. (2004) reported that the previous PB1-F2 knockout strategy including modification of the PB1-F2 start codon without alternative ORF and introduction of a stop codon downstream of initial 8 aa was not sufficient, because it allowed expression of the PB1-F2 C-terminal region from a downstream initiation codon. Therefore, a new strategy was used to knock out the expression of PB1-F2 and its downstream initiation products.

Viruses with or without mutation in PB1-F2 gene were tested in mice. The role of PB1-F2 in pathogenesis in mice depends on the type of virus. With highly pathogenic mouse-adapted virus such as wtAWSN/33 (WSN), there was no evident role of PB1-F2 in pathogenesis in mice as compared with the knockout WSNdelF2 virus. Subsequently, a WSN influenza virus model with an intermediate pathogenetic phenotype was created. This virus (WPW or WPWdelF2 virus) possessed a chimeric WSN-PR8 PB1 protein. The chimeric PB1-F2 better interacted with other components of WSN polymerase (PB2 and PA) and promoted more efficient viral replication as compared with WSN virus with PR8 PB1. When tested in mice, WPW virus induced significantly higher weight loss and mortality than its PB1-F2 knockout counterpart. However, the kinetics of viral clearance was different. While the PB1-F2 knockout virus could no longer be detected in the lungs after day 5, WPW virus was still present in the lungs on day 7. This suggests that the immune response to the wt virus was suppressed as compared to the mutant (Zamarin *et al*., 2006). Importantly, Coleman (2007) recently reported that PB1-F2 increased pathogenicity by disrupting alveolar macrophages.

7. Antibodies against PB1-F2 are induced following influenza infection

In our experiments, the presence of antibodies specific to PB1-F2 in human convalescent sera obtained after natural infection during influenza epidemics was demonstrated by ELISA, immunoprecipitation and indirect immunofluorescence. Positive sera reacted with all synthetic overlapping peptides corresponding to different parts of PB1-F2 molecule. Nevertheless, a peptide corresponding to the N-terminus of PB1-F2 showed a significantly reduced binding. We also detected specific PB1-F2 antibodies in sera of intranasaly infected mice (Krejnusová *et al.,* unpublished data). It is so far not clear whether specific PB1-F2 antibodies have any biological significance.

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