Mapping mutations in influenza A virus resistant to norakin

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To elucidate the mode of action of norakin against influenza A virus we sequenced the hemagglutinin gene of 11 norakin-resistant mutants Resistance was coupled with 1–3 amino acid exchanges The majority of mutations was localized in the HA2 polypeptide and was mostly associated with changes in charge or polarity of the amino acids. The amino acid substitutions are discussed in the context of the 3D structure of X31 hemagglutinin considered to be representative of the influenza hemagglutinins Most of the mutations appear to destabilize the pH 7 0 structure by distorting or destroying hydrogen bonds as well as salt-bridges which are responsible for intra- and intersubunit contacts, while others destabilize the location of the fusion peptide, facilitating conformational changes in the presence of the inhibitor.

Influenza A, Norakin resistance, Hemagglutinin

1. INTRODUCTION

There is a need for effective antiviral agents to prevent and to treat influenza and other respiratory infections. The anticholinergic anti-Parkinson drug, norakin, was described to inhibit the multiplication of different ortho- and paramyxoviruses like influenza A and B, measles [1,2] and RS virus [3].

For influenza A the major envelope glycoprotein hemagglutinin was identified as the target of norakin action [4]. As shown earlier, norakin inhibits the entry of influenza A virus into the cell [5] as well as virus-induced hemolysis and the conformational change of viral hemagglutinin at acidic pH [4].

To elucidate the mode of action of norakin, a series of norakin-resistant mutants of the highly norakin-sensitive strain A/FPV/Weybridge was isolated [2,6], and their hemagglutinin genes were sequenced. After sequencing the first 4 mutants it became clear that resistance to norakin is not determined by a distinct (single) mutation [6]. Amino acid substitutions can take place at different positions along the whole polypeptide.

Here we describe data of amino acid substitutions in the hemagglutinin of a further 7 norakin-resistant mutants. On the basis of the 3D structure of the hemagglutinin of strain X31 [7], the locations and possible consequences of the mutations were analysed.

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2. MATERIALS AND METHODS

2.1. *Virus infection*

Influenza A/FPV/Weybridge was kindly provided by Dr Y. Ghendon (Research Institute for Viral Preparations, Moscow, USSR). The norakin-resistant mutants were isolated following 3 plaque purifications on primary chick embryo cells (CEC) in the presence of 20 μg norakin per ml as described previously [2]. All strains were grown in the allantoic cavity of 11-day-old embryonated eggs. Virus titers and norakin sensitivities were determined by plaque tests on CEC.

2.2. *Virus RNA isolation*

The viruses were purified by repeated centrifugation on sucrose density gradients. Total viral RNA was isolated by the method of Hay et al. [8] and stored at −20°C.

2.3. *Nucleotide sequence analysis*

The hemagglutinin genes were sequenced using the dideoxynucleotide chain termination procedure of Sanger et al. [9] and Air [10] with minor modifications. The primers were complementary to nucleotides 5–20, 228–243, 426–438, 571–586, 831–846, 990–1005, 1112–1127, 1314–1329 and 1525–1540 according to the gene 4 sequence of A/FPV/Weybridge as determined by Hay et al (personal communication).

2.4. *Computer analysis*

Amino acid sequences were aligned according to Lipman and Pearson [11] with a gap penalty of 0.5. The total homology between the HAs of strain X31 [12] and strain Weybridge (Prosch et al., unpublished) was 41% for HA1 and 66% for HA2, respectively. Due to the high degree of homology, there is little doubt about the similarity of the 3D structures of X31 and Weybridge HA.

Ionic charge–charge interactions were counted in an all-or-none fashion, depending on whether the interacting groups of atoms were within a cutoff distance of 4 Å or not. Hydrogen bond interactions were treated similarly. The cutoff distance between the H-bond donor and acceptor was 2.8 Å.

The accessibility of each atom is defined according to Richards [13]. The computer program to calculate the accessible surface area

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Table I
Characterization of amino acid substitutions in the hemagglutinin

Mutant	Position[a,b] in subunit		Accessibility of side chain in %[d]	Type of secondary structure[e]	Change due to mutation			
	HA1	HA2			Ionic	Hydrophobicity[f]	Sec. structure probability[g]	Volume (Å^3)[h]
2		112 D⟶G	3	α	−⟶0	−1.77⟶0	1.01⟶0.57	74⟶44
15[c]		112 D⟶Y	3	α	−⟶0	−1.77⟶2.44	1.01⟶0.69	74⟶124
10		131 E⟶K	1	β	−⟶+	−1.46⟶−2.26	1.19⟶0.74	90⟶108
12[c]		134 G⟶D	25		0⟶−	0⟶−1.77		44⟶74
7	205 G⟶R		2[i]	β	0⟶+	0⟶−2.64	0.75⟶0.93	44⟶112
4	205 G⟶R		2[i]	β	0⟶+	0⟶−2.64	0.75⟶0.93	44⟶112
		114 E⟶D	8	α	−⟶−	−1.64⟶−1.77	1.51⟶1.01	90⟶74
5[c]	122 T⟶P		74	β	0⟶0	0.21⟶1.73	1.19⟶0.55	77⟶81
		41 T⟶A	8	α	0⟶0	0.21⟶0.40	0.83⟶1.42	77⟶61
1[c]	91 R⟶Y		61		+⟶0	−2.64⟶2.24		112⟶124
	210 H⟶N		7	β	+⟶0	−1.57⟶−0.28	0.87⟶0.89	99⟶78
6		36 A⟶S	3	α	0⟶0	0.40⟶−0.27	1.42⟶0.77	61⟶61
		50 G⟶V	25	α	0⟶0	0⟶1.46	0.57⟶1.06	44⟶91
16		107 T⟶A	0	α	0⟶0	0.21⟶0.40	0.83⟶1.42	77⟶61
		19 D⟶N	60		−⟶0	−1.77⟶−0.28	1.46⟶1.56	74⟶78
3	280 E⟶D		52		−⟶−	−1.46⟶−1.77		90⟶74
		83 W⟶C	5	α	0⟶0	2.55⟶0.90	1.08⟶0.70	144⟶72
		123 R⟶S	32	α	+⟶0	−2.64⟶−0.27	0.77⟶0.98	112⟶61

[a] Position of the amino acids according to X31 numbering
[b] Single letter code
[c] Results published previously [6]
[d] The mean accessibility of the whole HA protein is 24% of the maximal accessibility [14]
[e] According to Dictionary of Secondary Structure of Proteins (DSSP) [15]
[f] Scale according to the competition in charged amino acid side chains [16]
[g] Change of the secondary structure probability of the type observed in X31 [17]
[h] Molar volume of the residue [18]
[i] Accessibility of the total residue

Remarks: Probable structural consequences of the amino acid substitutions

Mutant 2 and 15:

Asp 112 of HA2 forms four hydrogen bonds to residues 1 and 3–6 of the N-terminal (fusion) peptide of HA2. Substitution of Asp by Gly or Tyr should destroy this interaction and thereby increase the mobility of the N-terminus, as well as destabilize the position of the fusion peptide in the pH 7.0 conformation.

Mutant 10:

Glu-131 of HA2 is buried in the HA2–HA2 interface, forming a hydrogen-bonded salt link as well as hydrogen bonds to Arg-163 and to Arg-127 of the same chain. Substitution of Glu-131 by the positive charged Lys should eliminate these interactions and weaken trimer stability.

Mutant 7 and 4:

Substitution of the small uncharged Gly-205 by the positive charged Arg should reduce the high mobility of the polypeptide chain in this region and possibly result in drastic conformational change

Glu-114 forms a salt bridge to Lys-117 in that part of the long helix of the same HA2 polypeptide which stabilizes the location of the fusion peptide of a second HA2 chain via a hydrogen bond. When exchanged for Asp, which is smaller by one C-atom, the H-bond might not be formed, resulting in a destabilization of the fusion peptide in the pH 7.0 conformation.

Mutant 5:

The substitution of Thr-122 by Pro may restrict the high flexibility of this region in the polypeptide chain.

Thr-41 of HA2 lies in the HA2–HA2 interface and forms hydrogen-bond to Thr-21 and Asp-37 of a second HA2 molecule which should be absent when Thr-41 is substituted by Ala.

Mutant 1:

In the HA1 of strain X31 position 91 is occupied by Ser which forms a hydrogen bond to Asp-271 of the same polypeptide chain. The Arg-91 in strain Weybridge may engage in a salt bridge to Asp-271. This should be destroyed by replacement of Arg by Tyr.

His-210 is buried in the interface between two HA1 polypeptides. It forms a salt bridge to Asp-101 and a hydrogen bond to Arg-220 of another HA1 molecule. Both interactions may be destroyed or at least weakened by the mutation to Asn.

Mutant 6:

Ala-36 of HA2 forms an intramolecular hydrogen bond with Asp-19 of the same HA2 polypeptide chain. Substitution by Ser should destroy this hydrogen bond, probably destabilizing the pH 7.0 conformation

Mutant 16:

Asp-19 is localized on the surface of the protein. The exchange for Asn would increase the net charge on the surface. (The hydrogen bond to Ala-36 (see above) would be preserved.)

Mutant 3:

Like Tyr-83 in strain X31, Trp-83 may be buried in the interface between two HA2 molecules and juxtaposed to Arg-85 of another subunit. Substitution by the smaller Cys would destroy any possible interaction in this contact region.

Arg-123 of HA2 interacts with Glu-120 of the same polypeptide chain via a hydrogen bond and a hydrogen-bonded salt link. These would be destroyed by the substitution, and the decrease of electrostatic charge should further destabilize intramolecular connections.

is described in detail in Baumann et al. [14]. The reference state for accessibility (%) for amino acid X is taken from the extended conformation of the oligopeptide: Gly-Gly-X-Gly-Gly.

3. RESULTS AND DISCUSSION

Norakin-resistant mutants contained one (5), two (5) or three (1) mutations in their hemagglutinin gene resulting in amino acid substitutions (Table I). The sites are distinct and distributed over the polypeptide chains of both subunits, being scattered in space throughout the structure of the oligomer. The majority of amino acid substitutions (12 from 18) were in the HA2 polypeptide (Table I, column 2). Nine of 11 mutants show at least one amino acid exchange resulting in a change of charge; however, not in a fixed direction (Table I, column 5). Amino acid substitutions in the hemagglutinins of mutants 5 and 6 involve a change of polarity.

Furthermore, not only the polarity or hydrophobicity is significantly altered by most mutations (Table I, column 6) but also the residue volume (Table I, column 8) and the propensity of each amino acid residue to stabilize the local secondary structure (Table I, columns 4 and 7). We observed several mutations which would tend to disrupt strong intra- and intermolecular interactions like salt bridges and H-bonds (Table I, remarks).

Compared with the mean accessibility (24%) of HA amino acid residues, at least one substituted amino acid residue of each mutant (with the exception of mutant 12) exhibits a very low accessibility (0–8%) (Table I, column 3). This is remarkable in view of the generally higher frequency of mutations at the protein surface. Interestingly, all nonaccessible side chains discussed here are parts of secondary structure elements (Table I, column 4), forming the interface between elements of the HA.

Thus, the secondary structure type should be conserved, but as a result of the mutations the stability/flexibility of the secondary structure elements may be altered, permitting distinct movements of such elements with respect to each other.

How do these mutations relate to the mode of action of norakin as an inhibitor of influenza virus penetration? They should allow the acid-dependent conformational change of the hemagglutinin – the prerequisite of fusion between viral and endosomal membranes [19] – in the presence of the inhibitor.

Some of the sites (91, 112, 114) where amino acid substitutions to norakin-resistance occur were also found by Daniels et al. [20] in mutants resistant to high amantadine concentrations. However, with the exception of the amantadine-resistant mutant 4Y and the norakin-resistant mutant 2, the individual amino acid exchanges are not identical and the degree of cross-resistance varies greatly between individual mutants [2] (Schroeder et al., in preparation). Whether these differences in molecular mode of action of norakin on the one side and high amantadine concentrations on the other are caused by indirect (e.g. pH) or direct drug effects on the target protein is currently under investigation.

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