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The Essential Tyrosine of the Internalization Signal in Lysosomal Acid Phosphatase Is Part of a β Turn

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Summary

For rapid endocytosis lysosomal acid phosphatase requires a Tyr-containing signal in its cytoplasmic domain, as do cell surface receptors mediating endocytosis and clustering in coated pits. To determine the structure of the internalization signal an 18 amino acid peptide representing the cytoplasmic tail of lysosomal acid phosphatase was analyzed by two-dimensional nuclear magnetic resonance spectroscopy. Part of the peptide, 5-PPGY-8, forms a well-ordered β turn of type I in solution. Our result and data on the structure of the endocytosis signal of the low density lipoprotein receptor reported by Bansal and Gierasch in the accompanying paper represent experimental determinations of the three-dimensional structure of protein transport signals and suggest that the essential aromatic amino acid of internalization signals is recognized by a putative cytoplasmic receptor in the structural context of a tight turn.

Introduction

Cell surface receptors mediating endocytosis and transcytosis, such as the receptors for transferrin, low density lipoprotein (LDL), polymeric immunoglobulin, and the cation-independent mannose 6-phosphate receptor, are clustered in clathrin-coated pits from where they are rapidly internalized (Goldstein et al., 1985). Receptor mutants lacking the cytoplasmic domain accumulate at the cell surface, suggesting that signals required for the rapid internalization are located in their cytoplasmic tails. Tyr residues are essential constituents of these internalization signals (Davis et al., 1987; Lehrman et al., 1985; Iacopetta et al., 1988; Jing et al., 1990; Mostov et al., 1986; Breitfeld et al., 1990; Lobel et al., 1989; Miettinen et al., 1989). The absence of additional sequence similarities suggests a conformational nature of the internalization signals, which are thought to be recognized by a common cytoplasmic receptor. The hydroxyl apatite II (HA-II) adaptor complex (Pearse and Robinson, 1984), which binds to clathrin and the cytoplasmic tails of cell surface receptors clustered in coated pits, is a likely candidate for a cytoplasmic receptor recognizing internalization signals (Pearse and Robinson, 1990).

Human lysosomal acid phosphatase (LAP) is synthesized as a glycoprotein with a single transmembrane domain separating a large luminal extracytoplasmic from a C-terminal cytoplasmic domain (Pohlmann et al., 1988). The cytoplasmic domain of LAP is known to harbor a Tyr-containing internalization signal functionally comparable to the signals found in cell surface receptors (Peters et al., 1990). Newly synthesized LAP is transported to the plasma membrane; it then recycles between endosomes and the cell surface before it is targeted to the lysosomal compartment (Braun et al., 1989). Within the lysosomes the luminal domain is released by proteolytic cleavage from the membrane anchor to form mature LAP (Gottschalk et al., 1989). The recycling between cell surface and endosomes depends on the Tyr-containing internalization signal, since deletion of the cytoplasmic tail or substitution of the Tyr by Phe causes an accumulation at the cell surface. Furthermore, transfer of the 19 amino acid cytoplasmic domain of LAP onto a resident membrane glycoprotein results in rapid endocytosis of the chimeric polypeptide, demonstrating that the short LAP cytoplasmic domain adopts a functionally active conformation in the absence of the LAP luminal and transmembrane domains (Peters et al., 1990).

Here we report conformational studies on a chemically synthesized 18 amino acid peptide representing the LAP cytoplasmic tail. We show that part of the peptide, 5-PPGY-8, adopts a β turn of type I in solution. Bansal and Gierasch (1991) describe a similar structure for the NPXY internalization signal of the LDL receptor. Taken together these two studies suggest that the essential aromatic amino acid residue of internalization signals is presented to a putative cytoplasmic receptor in the structural context of a tight turn.

Results

An 18 amino acid peptide representing the cytoplasmic tail of LAP (Table 1) was chemically synthesized and purified as described under Experimental Procedures. The three-dimensional (3D) structure of the tail peptide in aqueous solution was analyzed by 2D nuclear magnetic resonance (NMR) spectroscopy at 600 MHz. Complete proton resonance assignments were made for all residues (Table 1), and no strong 1D and 2D peaks were left unexplained.

Deuterium–hydrogen exchange rates for backbone amide protons were determined (Figure 1). At pD 3.1 all amide protons are exchanged in less than 11 min except those of Tyr^{8} (15 min) and Gly^{14} (23 min). Quantitative estimates of intrinsic exchange rates ruled out the possibility that sequence-dependent effects cause the observed variation in exchange rates (Molday et al., 1972). Therefore the slow

Table 1. Chemical Shifts (ppm) of the 18 Amino Acid LAP Tail Peptide in H_2O at pH 5.8, 292 K

	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	$C_\epsilon H$	$N_\epsilon H$
Met[1]		4.16	2.18	2.60		2.10	
Gln[2]	8.83	4.39	2.08 2.00	2.39			7.42^1 6.82^2
Ala[3]	8.58	4.28	1.37				
Gln[4]	8.42	4.58	1.96^3 1.81^2	2.27			7.63 6.93
Pro[5]		4.70	2.37^3 1.90^2	2.02	3.79^3 3.54^2		
Pro[6]		4.41	2.31^3 1.89^2	2.06	3.83^3 3.66^2		
Gly[7]	8.55	3.93^2 3.85^1					
Tyr[8]	7.94	4.46	3.02^2 2.96^3		7.06	6.78	
Arg[9]	8.10	4.22	1.71 1.62	1.48	3.14		7.19
His[10]	8.54	4.61	3.19 3.14		7.24	8.52	
Val[11]	8.22	4.07	2.02		0.91		
Ala[12]	8.50	4.32	1.39				
Asp[13]	8.33	4.58	2.69				
Gly[14]	8.41	3.98 3.92					
Glu[15]	8.22	4.31	2.05^2 1.91^3	2.27 2.21			
Asp[16]	8.45	4.57	2.65 2.60				
His[17]	8.23	4.69	3.29 3.21		7.31	8.55	
Ala[18]	8.29	4.15	1.37				

Stereospecific assignments are indicated by superscripts, e.g., 1 for $C_{\alpha 1}H$, 2 for $C_{\beta 2}H$, and 3 for $C_{\beta 3}H$ (IUPAC-IUB notation). Stereospecific assignments of C_β and N_ϵ proteins are based on intraresidue NOE patterns and $^3J_{H\alpha H\beta}$ coupling constants (Wagner et al., 1987); those of C_α and C_δ protons are based on conformational consistency.

rates are clear evidence for local structure at Tyr[8] and Gly[14].

Further evidence for local structure around Tyr[8] and Gly[14] comes from sequential NH–NH nuclear Overhauser effects (NOEs) linking Gly[7]-Tyr[8]-Arg[9] and Gly[14]-Glu[15]-Asp[16]. Medium range NOEs between $C_{\beta 3}H$ of Gln[4] and both C_β and C_δ protons of Tyr[8] as well as between $C_{\delta 2}H$ of Pro[5] and $C_{\beta 2}H$ of Tyr[8] define a turn involving Gln[4] to Tyr[8] (Figures 1 and 3), with the NH of Tyr[8] high field shifted by 0.24 ppm. Both Gln[4]-Pro[5] and Pro[5]-Pro[6] peptide units are in trans, as no sequential $C_\alpha H$–$C_\alpha H$ NOEs are detected. Adjacent to the turn region, an additional NOE is observed between

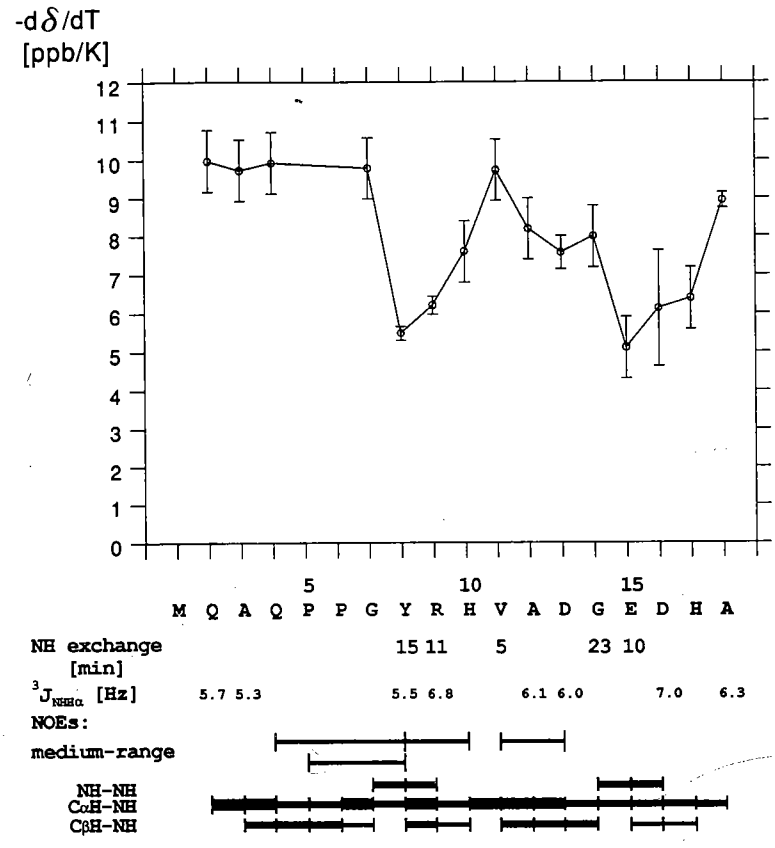


Figure 1. Temperature Shift of Backbone Amide Proton Resonances, Hydrogen–Deuterium Exchange Times, $^3J_{H\alpha NH}$ Coupling Constants, and NOEs

The datum point (with error bar) for the temperature shift of the backbone NH proton resonance of each residue (Gln[2] to Ala[18]) is the rate of change of chemical shift δ with temperature T (ppb/K) in H_2O. A straight line was fit to the δ(T) data for each residue, and the slope of this line is plotted here. The higher the temperature shift, the more exposed the proton is to solvent. Hydrogen–deuterium exchange times are in line "NH exchange"; where no time is given the exchange time was less than 11 min.

$^3J_{H\alpha NH}$ coupling constants were determined from the 1D spectrum for all residues with an NH peak sufficiently separated.

The observed "medium range," NH-NH, and other sequential NOEs are given in strip form. Atoms involved in medium range NOEs are listed in the text. Strong, medium, and weak NOEs are indicated by thick, medium, and thin lines, respectively. An additional NOE was observed between Met[1] $C_\beta H$ and Gln[2] $N_{\epsilon 2}H$. For other details, see Experimental Procedures.

Side chain temperature dependence was measured for Gln[2] ($N_{\epsilon 1}$ −5.22 ± 0.24, $N_{\epsilon 2}$ −4.76 ± 0.46 ppb/K) and Gln[4] ($N_{\epsilon 1}$ −6.65 ± 0.43, $N_{\epsilon 2}$ −5.88 ± 0.38 ppb/K); these are not shown in the graph.

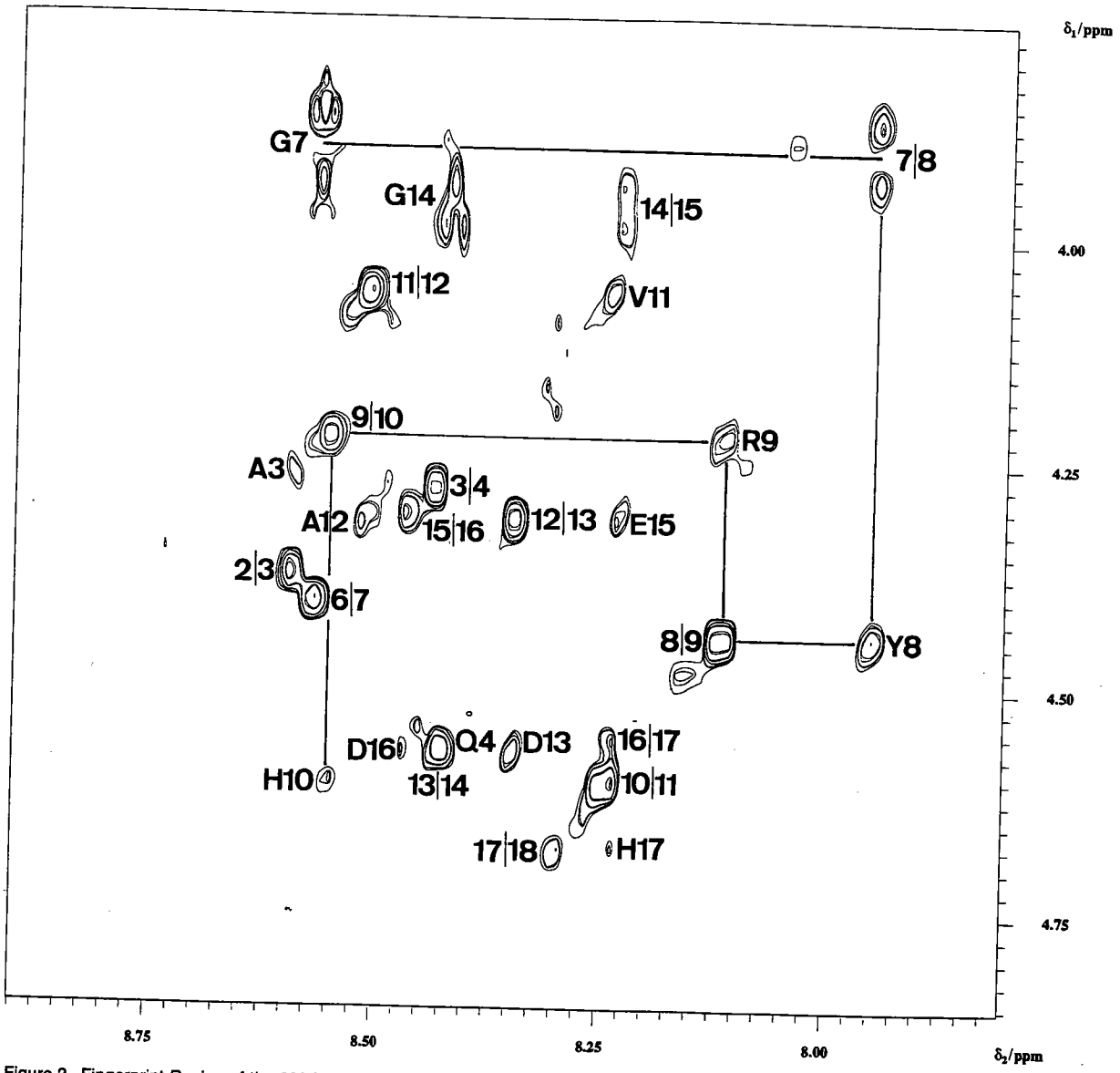
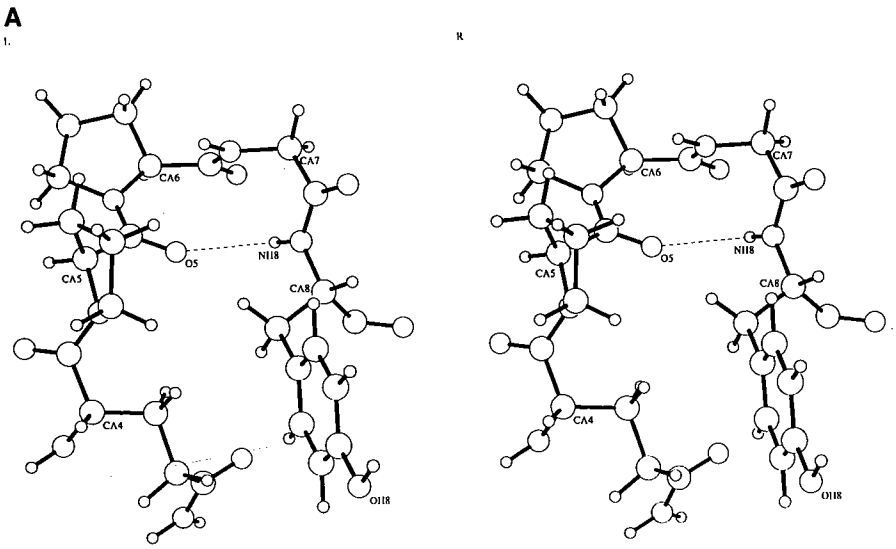
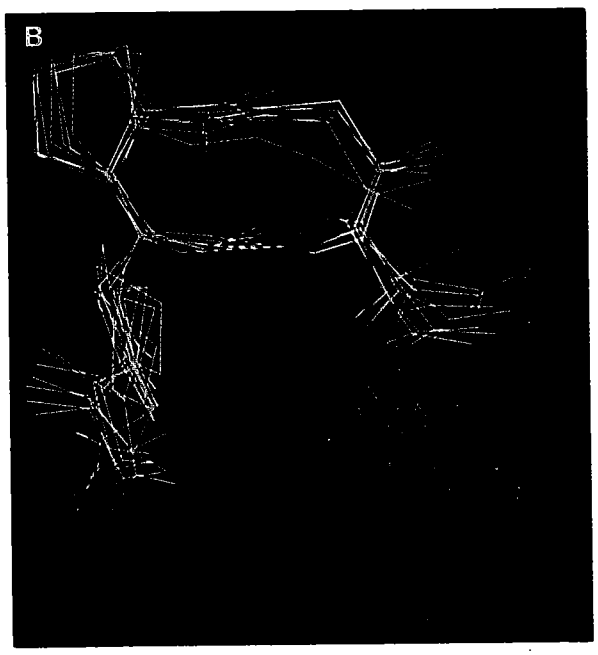


Figure 2. Fingerprint Region of the 600 MHz NOESY Spectrum (292 K [pH 5.8], t_{mix} = 250 ms)
Intraresidue NH–CαH NOEs are labeled with the one-letter amino acid code, sequential ones with the corresponding residue numbers (*i*/*i* + 1). For illustration, the connectivities from Gly[7] to His[10] are traced out. Successive contour levels differ by a factor of sqrt(2). For Gln[2] and Ala[18], the intraresidue NH–CαH NOEs occur below the lowest contour level shown here.

the C_ε protons of Tyr[9] and the C_α proton of His[10]. At Gly[14]-Asp[18] the observed NOEs do not allow us to define further the local structure.

In addition to the preferred β turn conformation some fraction of the total peptide is present in random structure. This is indicated by the fact that the intensity of all sequential C_αH–NH NOEs is higher than that of the intraresidue ones (Figure 2). In light of the occurrence of a single set of resonances, this is clear evidence for a dynamic equilibrium between extended random and nonrandom structure. The fraction of nonrandom structure can be estimated using measurements of the dependence of amide proton chemical shifts on temperature (Dyson et al., 1988; Sumner et al., 1990). If there are no major changes in conformational equilibrium, that dependence is expected to be linear, with maximum slope for completely solvent exposed protons and minimum slope for protected ones. For any particular NH proton, intermediate values are due either to partial shielding or to partial occupancy of the nonrandom state. The extent of partial occupancy can be estimated by linear interpolation between the extreme values. Taking the maximum value of −10 ppb/K from this experiment (Figure 1) and a minimum value of 0, we obtain an estimate of 50% for the fraction of nonrandom structure at Tyr[9]. This estimate is conservative for two reasons: even completely shielded protons typically have nonzero temperature dependence, e.g., −5.7 ± 0.3 ppb/K and −4.6 ± 0.3 ppb/K for two extremely slowly exchanging α helical





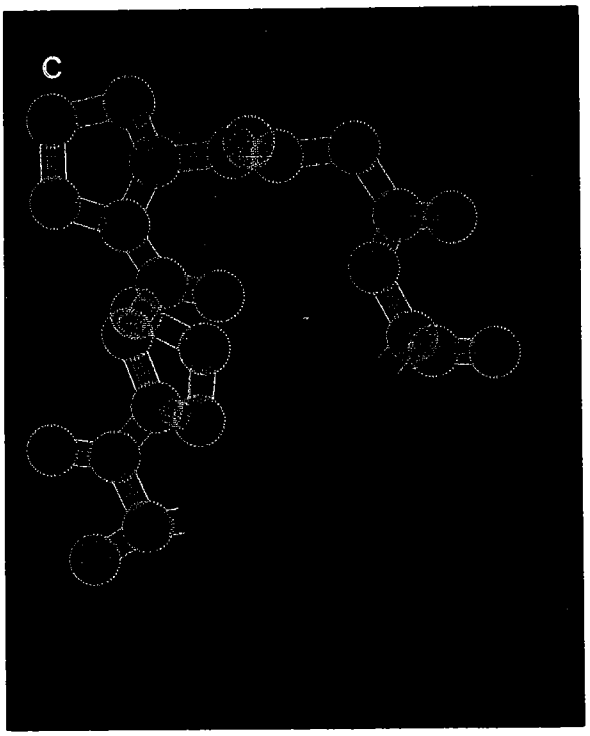


Figure 3. Structure of the 5-PPGY-8 Type I β Turn in the Cytoplasmic Domain of LAP

(A) Stereo view of one representative NMR structure (residues 4-QPPGY-8). The dotted line is the (i, i + 3) hydrogen bond Tyr^8NH-Pro^5O, a key interaction in β turns. The Pro^6-Gly^7 peptide unit is oriented as in a β turn of type I. The Tyr^8 OH group is essential for the endocytosis signal (see text). Stereo view produced using the program PLUTO.

(B) Color view of eight calculated NMR structures (4-QPPGY-8). The essential side chain of Tyr^8 is green (oxygen red) and that of the neighboring Gln^4 is red. The backbone hydrogen bond from NH (blue) of Tyr^8 to CO of Pro^5 is indicated by a dashed line. Note that the position of the Tyr side

NH protons in the rop protein at pH 7.2 (W. E., unpublished data); and NH of Tyr[8] may not be completely shielded from solvent in the ordered structure.

Conformational calculations using distance geometry and molecular dynamics (see Experimental Procedures) resulted in eight structures fully consistent with the structural NOEs, coupling constants, and NH exchange data. All eight computed structures (Figure 3B) have a type I β turn, with a hydrogen bond between the NH of Tyr[8] and the CO of Pro[5] (Figures 3A and 3B). No computed low energy structure consistent with the observed NOEs has a type II turn.

Discussion

The data presented here provide clear evidence for a tight-turn conformation at 5-PPGY-8 of the 18 amino acid peptide corresponding to the cytoplasmic domain of LAP. The type I β turn includes the aromatic amino acid residue Tyr[8] at position i + 3, previously shown to be an essential constituent of the internalization signal in the cytoplasmic domain of LAP (Peters et al., 1990).

The first indication for a tight-turn confirmation of internalization signals in receptors came from a computer search by Collawn et al. (1990). They observed that in proteins with known 3D structure, eight of the ten tetrapeptides most closely related to the YXRF internalization signal of the transferrin receptor and four out of five tetrapeptides with sequences related to the NPXY internalization signal of the LDL receptor have tight-turn conformations. In the accompanying paper Bansal and Gierasch (1991) show by NMR spectroscopy of nonapeptides that the NPXY internalization signal of the LDL receptor indeed adopts a type I β turn conformation in solution very similar to the structure described for the LAP internalization signal presented in this study.

A search for sequences with structures similar to those of the PPGY internalization signal of LAP in the Brookhaven Protein Data Bank was performed. Inspection of the resulting peptides by computer graphics revealed a GPGY tetrapeptide forming a type I turn in wheat germ agglutinin (data set 3WGA; Wright, 1987) and an APGY tetrapeptide forming a type II turn in trypsin (data set 1SGT; Read and James, 1988).

From the results of these NMR studies we conclude that an aromatic residue presented in the context of a tight turn appears to be a common structural motif of internalization signals. In LAP the immediate context of the aromatic residue Tyr[8] is provided by the side chains of Gln[4] and Pro[5], which are close to that of Tyr[8], and by the main chain of the β turn. The NOEs observed between one of the C_β protons of Gln[4] and the C_β and C_δ aromatic ring protons of Tyr[8] indicate that the side chains are in close proximity. It remains to be determined whether or not the side chain of Gln[4] participates in a specific interaction between the cytoplasmic tail of LAP and a cytoplasmic receptor along with the side chain of Tyr[8]. Preliminary results indicate that Gln[4] can be replaced by an Ala residue without impairment of internalization (C. P., unpublished data).

For LAP the aromatic residue of the internalization signal must be a Tyr, as replacement of Tyr[8] by Phe impairs internalization (Peters et al., 1990). This requirement does not hold for the internalization in the transferrin (McGraw and Maxfield, 1990) and the LDL receptor (Davis et al., 1987), as Tyr can be replaced by Phe or Trp without loss of internalization.

The interaction of cytoplasmic tails and receptors is likely to involve additional structural elements in the cytoplasmic tail, as it has been shown that amino acid residues at some distance from the Tyr contribute to the efficiency of internalization of several membrane glycoproteins (Chen et al., 1990; McGraw et al., 1991; Ktistakis et al., 1990). These residues can modulate the signal either by interacting directly with the receptor or by altering the conformation of the cytoplasmic tail. In analogy to other known cases of specific peptide–protein interaction (Bolognesi and Gatti, 1982; Knighton et al., 1991), we favor the former alternative. We therefore propose that recognition of the Tyr residue presented in the context of a tight turn is the essential recognition event contributing a significant fraction of the binding energy. Additional interactions of the receptor with other residues located outside the tight turn may help to establish the overall affinity required for efficient and rapid internalization.

Experimental Procedures

Peptide Preparation

The peptide representing the cytoplasmic tail of LAP (residues 406–423 in Pohlmann et al., 1988) was synthesized using 1-fluorenylmethoxycarbonyl (FMOC)-protection chemistry and PyBOP/HOBt amino acid activation (Coste et al., 1990) on a Milligen 9050 peptide synthesizer. Following synthesis the peptide was purified by preparative reverse-phase high pressure liquid chromatography and analyzed by high pressure liquid chromatography, UV spectroscopy, mass spectroscopy, and sequence analysis.

NMR Data Collection

Two-dimensional experiments were double quantum filtered coherence spectroscopy (DQF-COSY), nuclear Overhauser effect spectroscopy (NOESY) (t_{mix} = 250 ms), and total correlated spectroscopy (TOCSY) (t_{mix} = 75 ms), performed at 600 MHz. Solvent conditions for 2D experiments were as follows: 90% H_2O/10% D_2O (pH 5.8), 292 K, 10 mM peptide concentration, 100 mM NaCl, 100 mM phosphate buffer.

The temperature dependence of chemical shifts was determined

chain is more accurately defined by the NMR data than that of Gln[4]. The pairwise rms distance between equivalent atoms of Pro[5]-Tyr[8] was 0.57 ± 0.28 Å for backbone atoms and 1.14 ± 0.37 Å for all heavy atoms. The mean violation of the 33 distance restraints used in the calculation was 0.055 ± 0.011 Å.

The mean values and rms deviations (in parentheses) of dihedral φ, ψ, χ_1 angles are as follows. Gln[4]: undefined, 201(40), −67(12); Pro[5]: −93(13), 150(15), −16(29); Pro[6]: −4(8), −68(9), −48(2); Gly[7]: −116(18), 47(8), -; Tyr[8]: −93(35), −71(9), 187(10).

(C) Color view of one of the eight structures in (B).

The same color scheme as in (B) was used. The structural context of Tyr[8] is the entire β turn, with the chain of Gln[4] close to the essential OH of Tyr[8]. Color views by Gerrit Vriend (WHATIF).

from 2D DQF-COSY spectra at 285 K, 292 K, and 300 K (same conditions as in Table 1) for all residues and in addition from 1D spectra at 288 K, 296 K, 304 K, and 308 K for Tyr^8, Arg^9, Asp^{13}, and Ala^{18}. For Gln^2 only data at 285 K, 288 K, and 292 K were used. Error bars in Figure 1 are statistical 95% confidence intervals when seven data points were available, else they are estimated. Correlation coefficients for the linear fits were better than −0.9987 for the seven data points.

To determine deuterium exchange times, the peptide was prepared in H_2O, lyophilized, and redissolved in pure D_2O (pD 3.1, uncorrected pH meter reading), and a time series of 1D spectra was taken at approximately 2 min intervals. To determine the chemical shifts at this pD, a 2D DQF-COSY spectrum was taken in 90% H_2O/10% D_2O. Exchange times at pD 5.5 were about 1 min for Tyr^8 and Gly^{14}. The identification of NH of Tyr^8 as slowly exchanging was unambiguous, that of Gly^{14} required comparison of the exchange data at the two pD values; even then, there is a small probability that Asp^{16} is slowly exchanging instead of Gly^{14}, as their chemical shifts are close (Table 1).

Calculation of Structures

For structure calculation NOEs were grouped in three intensity classes. Distance ranges were estimated using the fact that intraresidue $C_\alpha H$–NH distances are always smaller than 2.8 Å. Selected distance ranges were <3.0 Å, <3.5 Å, and <4.0 Å. Restraints were derived from sequential NH–NH, $C_\beta H$–NH, and "medium range" NOEs. Nonobserved NOEs, Pro^6 $C_\gamma H$ and $C_\alpha H$ to Tyr^8 NH, were introduced as lower limits of 3.2 Å. Side chain χ_1 angles of residues 4, 8, and 15 were restrained to one of the three staggered values on the basis of intraresidue data. After preliminary DISMAN (Braun and Go, 1985) runs both Pro C_δ protons and Gly^7 C_α protons were assigned stereospecifically on the basis of conformational consistency and used in further calculations.

Out of 40 DISMAN starting structures the 12 best ones in terms of NOE and van der Waals violations were refined by 8 ps restrained molecular dynamics at 600 K, followed by 300 steps of energy minimization (XPLOR [Brunger, 1987], ε_0 = 4.0, quadratic NOE term, force constant = 30 kcal/Å^2). Four of these structures had no solvent-protected Tyr^8 NH proton, inconsistent with exchange rate and NH temperature shift data, and were discarded.

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