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Structural Domains of Chimeric Dopamine-Noradrenaline Human Transporters Involved in the Na^+- and Cl^--Dependence of Dopamine Transport

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ABSTRACT

Catecholamine transporters constitute the biological targets for several important drugs, including antidepressants, cocaine, and related compounds. Some information exists about discrete domains of these transporters that are involved in substrate translocation and uptake blockade, but delineation of domains mediating the ionic dependence of the transport remains to be defined. In the present study, human neuronal transporters for dopamine and noradrenaline (hDAT and hNET) and a series of six functional chimeras were transiently expressed in LLC-PK1 cells. Substitution of Cl^- by isethionate reveals that cassette IV (i.e., the region of the transporter encompassing transmembrane domain 9 through the COOH terminal) plays an important role in the Cl^-- dependence of the uptake. Substitutions of Na^+ and NaCl by $Tris^+$ and sucrose, respectively, demonstrate that three different segments scattered across the transporter are involved in the Na^+- dependence of the transport activity: cassette I (i.e., the region from the amino terminus through the first two transmembrane domains), cassette IV, and junction between transmembrane domains 3 to 5 and 6 to 8. Results of the present work also suggest that the use of $Tris^+$ as a substitute for Na^+ results in a biased estimate of the Hill number value for hDAT. This study provides useful clues for identifying specific residues involved in the uptake function of the catecholamine transporters.

Dopamine and noradrenaline transporters (DAT and NET) are members of a family of Na^+ and Cl^--dependent transporters that mediate a rapid removal of neurotransmitters from the synaptic cleft. The major involvement of the DAT in terminating neurotransmission has been demonstrated clearly by gene disruption experiments (Giros et al., 1996; Jones et al., 1998). DAT and NET are predicted to have 12 transmembrane domains (TMDs) and to share a high amino acid sequence identity (Amara and Kuhar, 1993; Giros and Caron, 1993; Miller et al., 1997). They are involved in various diseases that result in high social, medical, and economic costs. The NET is a pivotal target for many antidepressant drugs (Richelson and Pfenning, 1984 Baldessarini, 1995). Drugs displaying a low selectivity for NET or DAT, such as amphetamine, methylphenidate, and pemoline, are efficient for reversing clinical symptoms of narcolepsy and attention deficit hyperactive disorder (Heiligenstein et al., 1996). Molecular genetic studies of humans have suggested that mutations in the DAT gene could be associated with attention deficit hyperactive disorder and other diseases, such as generalized anxiety, social phobia, and Tourette's disorder (Rowe et al., 1998; Swanson et al., 1998). In addition, a variety of reports demonstrates that the DAT constitutes an important yet nonexclusive target for the reinforcing properties of cocaine and related drugs (Kuhar et al., 1991; Rocha et al., 1998). Consequently, a better knowledge of the mechanisms underlying DAT and NET functioning remains a primary goal.

Certain transporters have been shown to display ion channel-like electrical activities, mediating both a constitutive leak current and a transport-associated current (Sonders et al., 1997). Thus, it seems that a thermodynamically uncoupled component makes a major contribution to the dopamine transport-associated current. Only few Na^+ and Cl^- ions are thermodynamically coupled to one or more steps of the transport activity of DAT and NET (i.e., binding of the substrate to the transporter, internalization, and reorientation of the transporter) (Sonders et al., 1997).

Despite their high amino acid sequence identity, catechol-

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ABBREVIATIONS: DAT, neuronal transporter of dopamine; NET, neuronal transporter of noradrenaline; TMD, transmembrane domain; hNET, human neuronal transporter of noradrenaline; GBR 12783, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)piperazine.

amine transporters are generally reported to have different stoichiometries for Na^+/Cl^-/substrate cotransport (i.e., 1:1:1 for NET and 2:1:1 for DAT; Friedrich and Bönisch, 1986; Krueger, 1990; McElvain and Schenk, 1992; Gu et al., 1994; see also Pifl et al., 1997). Nevertheless, this point calls for two remarks. First, as stated in a recent review, ion-dependence measurements performed in these studies give information only about the binding of the cosubstrates and they constitute at best an indirect estimate of the transport stoichiometry (Rudnick, 1998). Second, the use of inhibitory cations as substitutes for Na^+ in DAT transport studies could result in a biased estimate of the stoichiometry. NET and DAT can also be distinguished by the ionic concentrations that half-maximally stimulated their uptake activity. Results obtained using LLC-PK1 cells stably expressing these transporters indicated that the Na^+ K_m value for hNET was higher than that for rat DAT (Gu et al., 1994). This work also demonstrated that the Cl^- K_m value was markedly lower for NET than for DAT, in agreement with other studies (Friedrich and Bönisch, 1986; McElvain and Schenk, 1992; Pifl et al., 1997). These differences are not likely to be the consequence of different intracellular ionic media because two of the previous studies were performed on transporters expressed in the same cell hosts (Gu et al., 1994; Pifl et al., 1997).

Studies of functional chimeras have allowed the assignment of pharmacological and kinetic features of the uptake to discrete domains of DAT and NET (Giros et al., 1994; Buck and Amara, 1994, 1995). From these studies, it seems that the segment from the amino terminus through the first three TMDs is probably involved in the affinity for substrates and inhibitors, whereas TMDs 5 to 8 contribute to substrate translocation and affinity-selectivity for inhibitors. In addition, a region spanning TMDs 10 to 11 seems to be important for stereoselectivity and affinity for substrates (Buck and Amara, 1994, 1995; Giros et al., 1994).

In the present work, parental transporters of human origin (hDAT and hNET) and six functional chimeras generated by Giros et al. (1994) (Fig. 1; see also Fig. 4) were transiently expressed in LLC-PK1 cells. The ionic dependence of the [^{3}H]dopamine uptake operated by these cells was studied in media in which Na^+, Cl^-, or NaCl were substituted by $Tris^+$, isethionate, or sucrose, respectively.

Materials and Methods

Cell Culture. Parental and transiently transfected LLC-PK1 cells were maintained on plastic tissue culture dishes (Falcon, Oxnard, CA) in a Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Le Perray en Yvelines, France) and 50 mg/l of gentamicin at 37°C and 5% CO_2.

Preparation of Chimeras and Transfection. Chimeras between hDAT and hNET were constructed by homologous religation of the four cassettes produced by digestion of each of the cDNAs at three common restriction sites (Giros et al. 1994) (Fig. 1). Cassettes I to IV corresponded to the following segments: I, from the amino terminus to the intracellular loop 1 (nucleotide 399 in hDAT); II, from the end of cassette I to the end of the fifth TMD (nucleotide 852 in hDAT); III, from the end of cassette II to the medial part of the fourth intracellular loop (nucleotide 1303 in hDAT); and IV, from the end of cassette III to the carboxyl terminus (Fig. 1). cDNAs encoding for hDAT, hNET, and chimeric transporters were subcloned into vector pRC/CMV, which has a bacteriophage T7 promotor sequence and enhancer/promotor sequences from immediate early gene of human cytomegalovirus. LLC-PK1 cells were transiently transfected by the DEAE-dextran method (Promega, Charbonnières, France), according to manufacturer's procedures and using 1 to 5 μg of plasmid DNA. The transfection yield was enhanced by a glycerol shock step (15% final concentration). Parental LLC-PK1 cells have been reported not to display any detectable [^{3}H]dopamine uptake (Gu et al., 1994).

Uptake Experiments. LLC-PK1 cells transiently expressing the parental and chimeric transporters were grown in 24-well plates at 37°C. Forty-eight to 72 h after transfection, cell cultures were aspired free of medium and washed with 1 ml of incubation medium. PBS incubation medium (109 mM NaCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM Na_2HPO_4, 5.4 mM glucose, pH 7.4 $\pm$ 0.1) was used for NaCl- and Na^+-dependence studies. Na^+ and NaCl were substituted with equimolar concentrations of $Tris^+$ and twice-equimolar concentrations of sucrose, respectively. Uptake experiments performed with LLC-PK1 cells expressing hDAT have shown that a substitution of Na^+ by $Tris^+$ produced an uptake reduction which was less

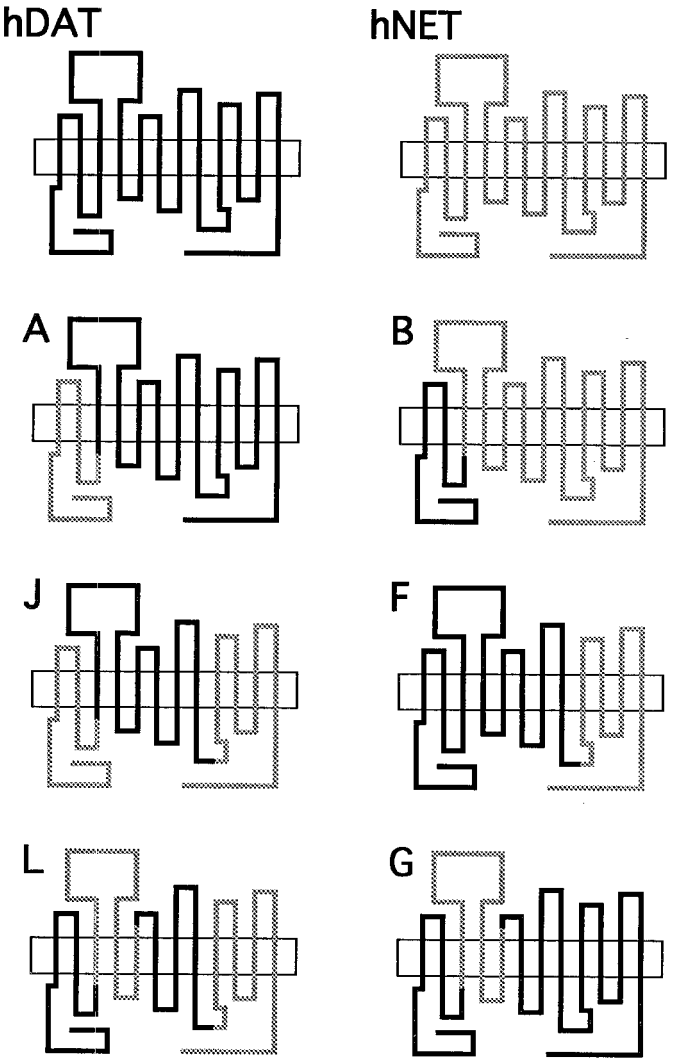

Fig. 1. Wild-type and chimeric catecholamine transporters. Twelve hydrophobic domains are modeled as membrane-spanning domains. Six functional chimeras between hDAT (black) and hNET (gray) were constructed by homologous religation of the four cassettes produced by digestion of the cDNAs at three common restriction sites: *Bgl*II (nucleotide 399 in hDAT), *Bst*EII (nucleotide 852 in hDAT), and *Cla*I (nucleotide 1303 in hDAT). Further details can be found in the legend to Fig. 4.

marked than a substitution by choline$^+$ or Li$^+$; the replacement of NaCl by sucrose produced a decrease in uptake of the same intensity than that produced by Tris$^+$.

For Cl$^-$-dependence experiments, the incubation medium was a modified Krebs-Ringer medium containing 109 mM NaCl, 27 mM $NaHCO_3$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 5.4 mM glucose, pH 7.4 ± 0.1. NaCl was substituted by equimolar concentrations of sodium isethionate. Cells were preincubated for 5 min at 37°C in 480 μl of incubation medium. Thereafter, 20 μl of [^{3}H]dopamine was added to obtain a final concentration of 50 nM. Preliminary experiments of saturation in PBS and modified Krebs-Ringer medium indicated that this concentration corresponds to 0.02 to 0.05 of the K_m values of the transporters for dopamine. After 5 min of incubation (except when indicated), the uptake was stopped by adding 1 ml of ice-cold incubation medium containing 10^{-5} M mazindol or 10^{-4} M cocaine. After aspiration, cells were carefully washed three times with the same ice-cold medium and then dissolved in 500 μl of 1N NaOH (1 h). The accumulated radioactivity was determined by liquid scintillation counting (Betamatic; Kontron Intertechnique, Plaisir, France). The specific uptake was calculated by subtracting the nonspecific accumulation of radioactivity in the presence of 10 μM to 1 mM cocaine according to the sensitivity of chimeras to the uptake blocker (Giros et al., 1994). The specific uptake was expressed as picomoles of dopamine per 10^6 cells per minute. [^{3}H]Dopamine was used as a unique substrate for uptake experiments because it is more stable than [^{3}H]noradrenaline; its K_m value for the uptake operated by both parental transporters is lower than that of noradrenaline (Gu et al., 1994); finally, differences in results caused by specific interactions of noradrenaline or dopamine with transporters and ions are thus discarded.

Chemicals. [^{3}H]Dopamine (10–20 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Saclay, France). Desipramine HCl was obtained from Ciba-Geigy (Rueil-Malmaison, France). Solutions (10 mM) of mazindol (Sandoz, Courbevoie, France) were prepared in 0.1 M HCl. Millimolar solutions of GBR 12783 diHCl (synthesized by Prof. M. Robba, Unité de Formation et de Recherche de Pharmacie, Caen, France) were prepared in distilled water. Subsequent dilutions and solutions of other reagents were made in incubation medium.

Calculations. For Na$^+$-dependence experiments, K_m, V_{max}, and Hill values were determined using a nonlinear least-squares fits (Origin; MicroCal Software, Northampton, MA) using the generalized Michaelis-Menten equation, $V = V_{max}\,[S]^n/(K_m{}^n + [S]^n)$, in which V is transport velocity, [S] is ion concentration, $1/K_m$ is the apparent affinity for ions, and n represents the Hill coefficient, assuming an involvement of n independent Na$^+$ of equal affinity. In Cl$^-$ dependence studies, K_m and V_{max} were determined using Lineweaver-Burk analysis (Origin curve-fitting program), because saturations were sometimes barely achieved, giving unreliable results when the aforementioned equation was used. The Cl$^-$-independent component of transport was subtracted for hNET B, -F, -G, -J, and -L. Demonstration of rather different K_m values for Na$^+$ and Cl$^-$ precluded the use of the aforementioned equation for experiments in which NaCl was substituted with sucrose. A stimulating concentration 50% (SC_{50}) corresponding to the NaCl concentration that half-maximally stimulated the dopamine uptake was calculated using the Ligand software (Biosoft, Milltown, NJ). The significance of changes was tested using a two-way analysis of variance with changes and transporters as factors.

Results

Pharmacological Study. A brief pharmacological study was carried out to ascertain that the blockade of the dopamine transport operated by LLC-PK1 cells transiently expressing hDAT and hNET by inhibitors displayed the expected selectivity. Addition of increasing concentrations of desipramine or GBR 12783 to the PBS during preincubation and incubation periods resulted in concentration-dependent inhibitions of uptake. The specific uptake of [^{3}H]dopamine by LLC-PK1 hDAT cells and LLC-PK1 hNET cells was blocked more selectively by GBR 12783 (IC_{50}, 180 ± 40 nM) and desipramine (IC_{50}, 27 ± 7 nM), respectively (means ± S.E.M. of three to four experiments performed in duplicate). In contrast, the transport activity was weakly inhibited by desipramine (IC_{50}, 22.6 ± 3.8 μM) in hDAT expressing cells and by GBR 12783 (IC_{50}, 1.45 ± 0.6 μM) in hNET cells.

The [^{3}H]dopamine uptake activity in a control medium containing 109 mM NaCl differed according to the transporter that was tested. Generally, rates of dopamine transport were higher for hDAT and hNET than for chimeras A, B, and F. The transport activity of the chimeric transporters J,

TABLE 1

Characteristics of the ionic dependence of the [^{3}H]dopamine uptake operated by hDAT, hNET, and six chimeric transporters.

Cl$^-$ ions contained in the Krebs Ringer medium were substituted by equimolar concentrations of isethionate (Cl$^-$ experiments), whereas Na$^+$ and NaCl contained in PBS were substituted with equimolar concentrations of Tris$^+$ (Na$^+$ experiments) or twice-equimolar concentrations of sucrose (NaCl experiments). Data are means ± S.E.M. of three to six estimates of K_m for ions and V_{max} of uptake calculated using either Origin (Na$^+$; Cl$^-$) or Ligand (NaCl) analysis software from data exposed in Fig. 2 and 3. V_{max} values are expressed as picomoles of [^{3}H]dopamine per 10^6 cells per minute. For NaCl experiments, we calculated an SC_{50} value that corresponds to the NaCl concentration that half-maximally stimulated the dopamine uptake.

Transporter	Cl$^-$			Na$^+$			NaCl		
	K_m	V_{max}	Hill number	K_m	V_{max}	Hill number	SC_{50}	V_{max}	Hill number
	mM			*mM*			*mM*		
hDAT **1234**	53 ± 12[b]	6.65 ± 1.1	0.84 ± 0.06	58 ± 7	2.8 ± 0.6	2.45 ± 0.25[d,e]	127 ± 54	10.7 ± 3.0	1.66 ± 0.17[f,g]
hNET 1234	27 ± 3[a]	6.3 ± 0.7	0.82 ± 0.20	100 ± 49	3.0 ± 1.55	1.31 ± 0.22	78 ± 17	17.4 ± 4.8	1.72 ± 0.38[f,g]
A **1234**	71 ± 7[a]	6.4 ± 2.2	0.97 ± 0.10	79 ± 35	1.3 ± 0.5	1.28 ± 0.32[e]	79 ± 19	2.6 ± 1.0	1.90 ± 0.28[f,g]
B 1234	26 ± 9	6.3 ± 1.9	0.55 ± 0.05	80 ± 14	1.2 ± 0.3	1.95 ± 0.26[d]	88 ± 18	5.7 ± 0.1	1.60 ± 0.20[f,g]
F **1234**	24 ± 3[b,c]	0.5 ± 0.1	1.12 ± 0.16	60 ± 18	4.4 ± 1.8	1.61 ± 0.25[d]	26 ± 3	5.0 ± 1.1	1.39 ± 0.27[f,g]
G **1234**	58 ± 29	0.05 ± 0.02	0.90 ± 0.26	84 ± 2	0.1 ± 0.1	4.69 ± 1.55	91 ± 14	0.1 ± 0.03	3.65 ± 2.60
J **1234**	24 ± 3[a,c]	0.2 ± 0.1	1.71 ± 0.90	30 ± 18	0.4 ± 0.1	0.92 ± 0.26	64 ± 26	1.51 ± 0.5	1.06 ± 0.23[f,g]
L **1234**	8.6 ± 0.2[c]	0.1 ± 0.05	1.35 ± 0.59	215 ± 48	0.9 ± 0.6	7.17 ± 5.16	198 ± 70	2.7 ± 1.35	1.48 ± 0.30

[a] No significant difference for the exchange of cassette I in A/hDAT, hNET/B, and J/F, with (F(1,17) = 0.98).
[b] No significant difference for the exchange of cassette II in hDAT/G and F/L, with (F(1,13) = 0.06).
[c] $P < .01$ for the exchange of cassette IV in F/hDAT, J/A, and L/G, with (F(1,18) = 8.35).
[d] $P < .001$ for the exchange of cassette I in hDAT/A, B/hNET, and F/J, with (F(1,16) = 15.7).
[e] $P < .01$ for the exchange of cassette IV in hDAT/F and A/J, with (F(1,10) = 5.6); the effect of the exchange of cassette IV was higher for hDAT/F than for A/J (F(1,10) = 12.6, $P < .01$).
[f] $P < .05$ compared with Hill values in Tris substitution for hDAT, B, and F, with F(1,15) = 4.75; no significant difference with Hill values in Tris substitution for hNET, A, and J, with F(1,16) = 3.33 ($P = .088$).
[g] No significant difference with Hill values in Tris substitution for hDAT, A, and hNET, B, F, and J, with F(1,38) = 0.007.

L, and G never exceeded 30, 15 and 4% of that of hDAT, respectively (Table 1).

Domains Involved in the Cl^--Dependence (Substitution by Isethionate). The uptake operated by hDAT and hNET differed in two ways. First, the transport activity of hNET displayed a Cl^--independent component, which represented 20% of the V_{max} value (Fig. 2); this component was subtracted for calculations of Cl^- K_m. Second, as evidenced

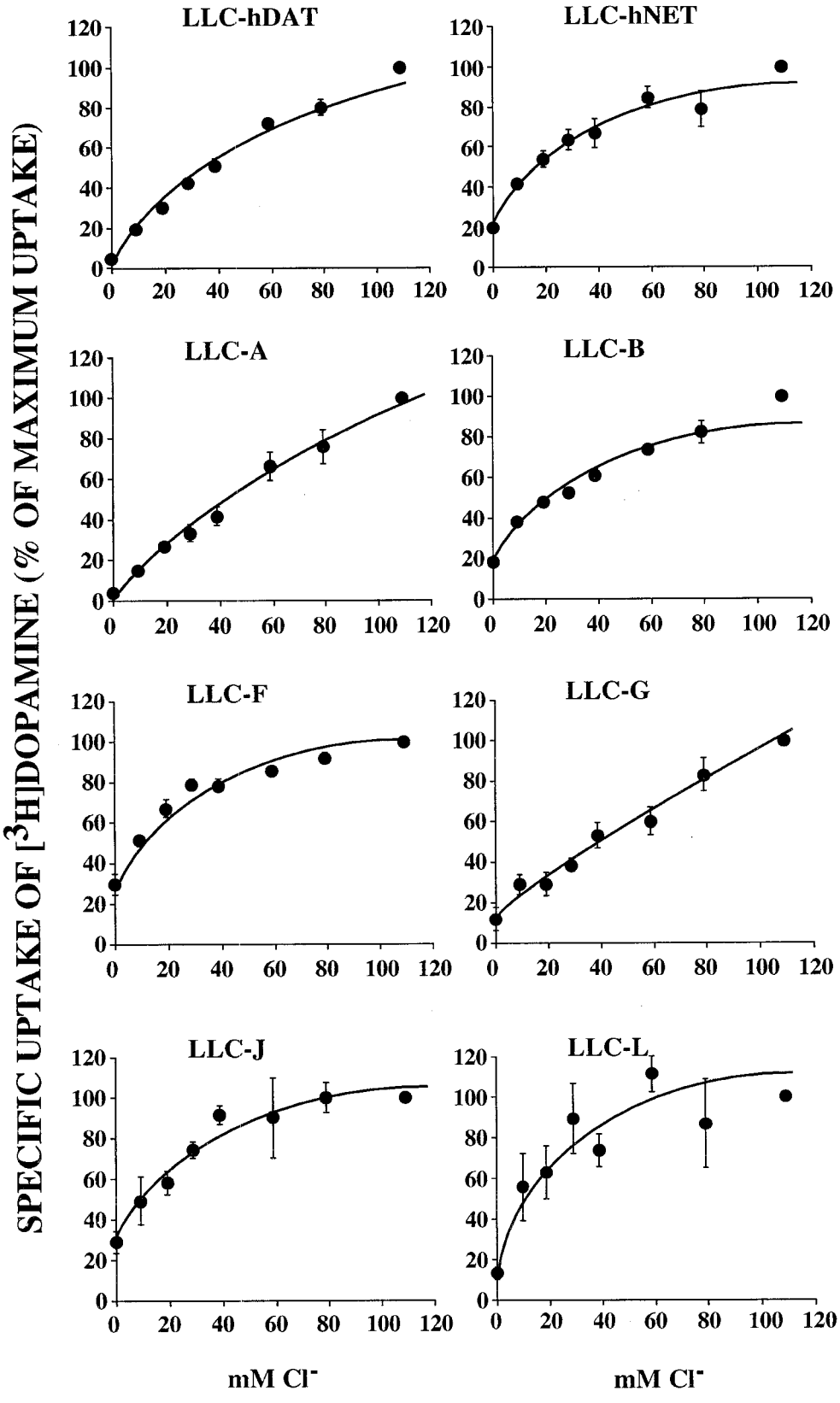

Fig. 2. Cl^--dependence of the [^{3}H]dopamine uptake operated by LLC-PK1 cells transiently expressing hDAT, hNET, or a chimeric transporter. Transport rates were measured using 5-min incubations, except for the chimeric transporter L (2 min). The Krebs Ringer medium was modified by substituting NaCl with equimolar concentrations of sodium isethionate. Uptake values are expressed as percentages of maximal uptake values, which are presented in Table 1. Data are means ± S.E.M. values from three to six independent experiments performed in duplicate.

by Cl^- K_m values, hDAT required higher concentrations of Cl^- to reach its maximal level of transport than hNET (Table 1).

Exchange of the first cassette of the transporters did not modify the Cl^- dependence of the [^{3}H]dopamine uptake: there was no significant difference in Cl^- K_m values between transporters resulting from this exchange, namely hNET and B, A and hDAT, and J and F (Table 1). This exchange was also without effect on the presence or the absence of a Cl^--independent component of uptake (Fig. 2). However, it is worth noting that chimeras F, G, J, and L exhibited such low transport activities that the comparison of their Cl^--independent transport component with that of other transporters became limited. Introduction of the second cassette of hNET in the backbone of hDAT and F gives chimeras G and L, which did not significantly modify the K_m values for Cl^- (Table 1).

On the contrary, insertion of cassette IV of hNET resulted in a significant decrease in Cl^- K_m values, as demonstrated by the following pairs of transporters, hDAT/F, A/J, and G/L (Table 1). Cl^- K_m values for chimera resulting from this insertion (F, J, and L) were in the range of the 10 to 20 mM concentrations.

Domains Involved in the Na^+-Dependence (Substitution by $Tris^+$). The transport activity of hNET was a simple hyperbolic function of the Na^+ concentration, in keeping with a Hill value close to unity (Fig. 3; Table 1). On the contrary, the sigmoidal shape of the Na^+-dependent transport operated by hDAT suggested that more than one Na^+ ion was involved in this transport process (Hill value, 2.45). Assuming that the different Na^+ ions were independent and of equal affinity, the Na^+ K_m values were 100 and 58 mM for hNET and hDAT, respectively.

Exchange of the NH_2 terminal part of the transporters produced a marked change in the Na^+ dependence of the transport. Thus, the introduction of the first cassette of hNET in a transporter resulted in a highly significant reduction of Hill number values, as observed for the pairs of transporters hDAT/A, B/hNET, and F/J (Table 1, Fig. 3). Hill values were $\geq$ 1.61 for transporters containing the NH_2-terminal part of hDAT, whereas they were in the range of unity ($\leq$ 1.31) for those containing the corresponding part of hNET.

Introduction of only cassette II of hNET in the backbone of hDAT or F, giving chimeras G and L, produced a dramatic modification in the shape of Na^+-dependence of the [^{3}H]dopamine transport, leading to major increases in Hill values (Fig. 3).

Another point raised by the present study is the possible involvement of the COOH-terminal part of the protein in the Na^+-dependence of the transport, because introduction of cassette IV of hNET in hDAT and A seemed to lower the cooperativity, giving Hill values of 1.61 and 0.92 for F and J, respectively (Table 1).

Substitution of NaCl by Sucrose. Replacement of NaCl by twice-equimolar concentrations of sucrose generated curves of ion-dependent uptake that generally matched rather well with those resulting from a substitution of Na^+ by $Tris^+$, except for chimeric transporters F and G (Fig. 3). Replacement by sucrose produced a decrease in their Hill values and a marked reduction in the ionic concentration that half-maximally stimulated the transport activity of F.

A further comparison of the results suggested that Hill number values obtained in these two sets of experiments could differ as a function of the origin of the first cassette. Thus, compared with a substitution by $Tris^+$, substitution of NaCl by sucrose significantly decreased the Hill value when cassette I of hDAT was present in the parental transporter and in chimeras B and F. On the other hand, the presence of cassette I of hNET in hNET, A, and J tended to increase Hill values in sucrose substitution experiments (Table 1).

Hill values obtained in these two sets of experiments demonstrated no significant changes as a function of the origin of the COOH-terminal part of the transporter (Table 1).

Discussion

The selectivity of the inhibition produced by desipramine and GBR 12783 confirms that [^{3}H]dopamine uptake was effectively catalyzed by hNET and hDAT, respectively. Desipramine preferentially blocked the uptake catalyzed by hNET and was markedly less potent on hDAT (Richelson and Pfenning, 1984; Gu et al., 1994), whereas GBR 12783 was 8-fold more potent on hDAT than on hNET. High-affinity GBR derivatives generally impair the dopamine uptake at low nanomolar concentrations (Van der Zee et al., 1980; Bonnet and Costentin, 1986; Giros et al., 1991; Buck and Amara, 1995). This is not the case in the present study, but previous works have already reported interactions of some of these compounds with hDAT at high nanomolar, and even (sub) micromolar, concentrations (Allard et al., 1994; Pristupa et al., 1994; Staley et al., 1994; Little et al., 1995).

Various features of the ionic dependence distinguished hDAT from hNET. First, the Cl^- concentration that half-maximally stimulated the dopamine uptake was markedly lower for hNET than for hDAT (Table 1). Second, hNET, in contrast to hDAT, displayed a transport activity that was partly independent upon Cl^- ions (Fig. 2). This apparent Cl^--independence can simply reflect the ability of isethionate to partially replace Cl^- in the transport process. Third, the use of $Tris^+$ as a substitute gave curves of Na^+-dependent uptake with a higher Hill value for hDAT than for hNET (Table 1). These Hill number values generally agree with those reported elsewhere (Friedrich and Bönisch, 1986, Krueger, 1990; Amejdki-Chab et al., 1992b; McElvain and Schenk, 1992, Gu et al., 1994; Pifl et al., 1997). However, it is noticeable that similar Hill number values were found for both hNET and hDAT when sucrose and isethionate were used as substitutes for NaCl and Cl^-. Consequently, if sucrose were essentially inert in the uptake process, a similar Hill number value would be expected for the Na^+-dependence of the transport operated by both transporters. This suggests that $Tris^+$ inhibited the transport operated by hDAT with an intensity sufficient to artifactually modify the Hill number value. Some previous studies had already raised this point (Shank et al., 1987; Amejdki-Chab et al., 1992a).

More generally, a comparison of results from $Tris^+$ and sucrose experiments evidenced that changes in Hill number values were linked to the origin of cassette I. Lower Hill number values were observed in sucrose experiments when transporters included the first cassette of hDAT, showing that it is involved in the inhibition of the transport activity produced by $Tris^+$ in hDAT, B, and F. On the contrary, $Tris^+$ could exert some stimulatory effect on the uptake when the

first cassette of hNET was present, as suggested by the tendency to increase Hill number values in sucrose experiments for hNET, A, and J.

These results are consistent with the hypothesis of a pivotal role of the highly conserved sequence included in the NH_2-terminal part of the neuronal transporters in their interactions with ions (Pacholczyk et al., 1991; Giros et al., 1994). A recent study in which the conserved Asp 98 of the first TMD of the serotonin transporter was mutated reached similar conclusions (Barker et al., 1999). In agreement with

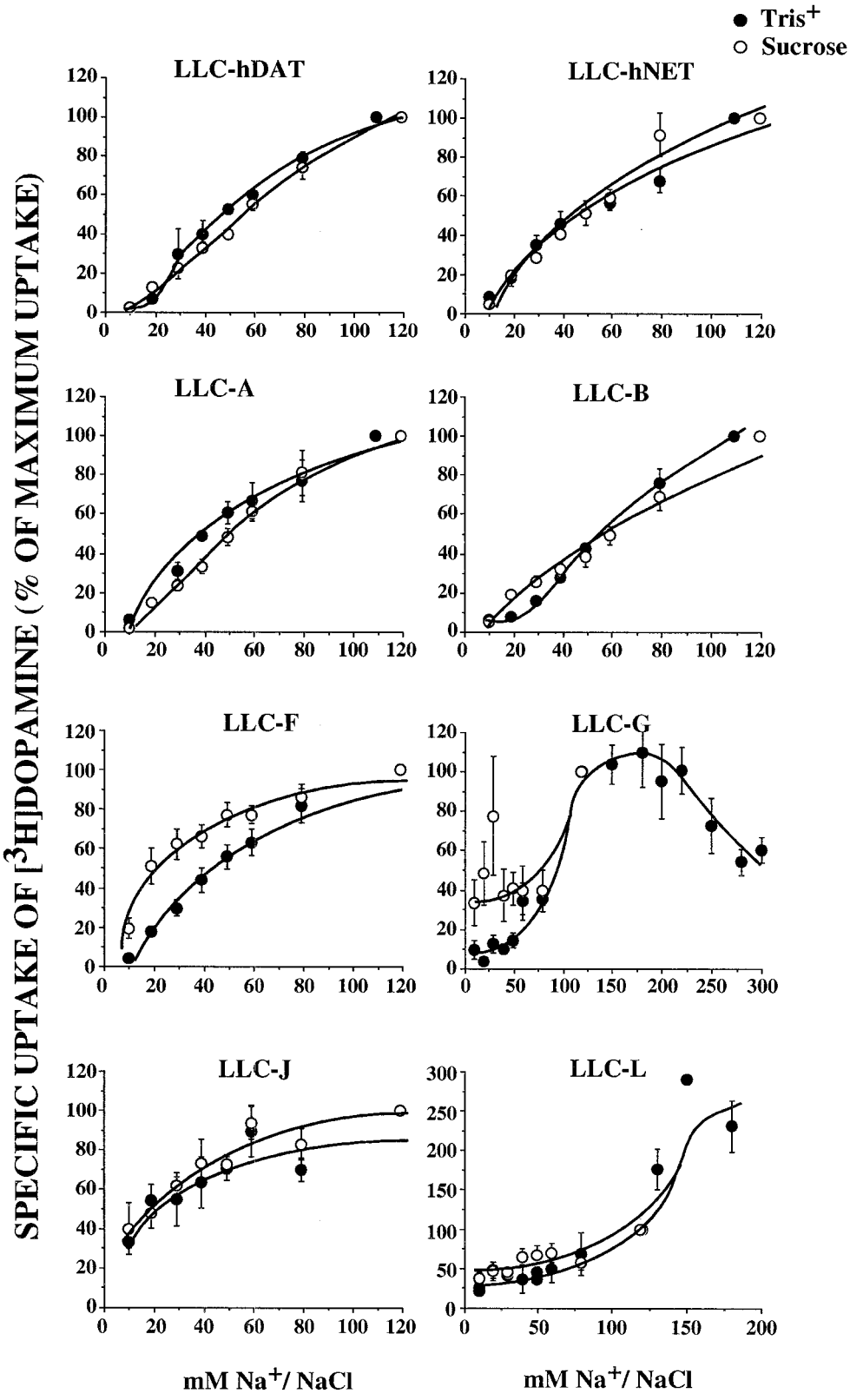

Fig. 3. NaCl- and Na^+-dependence of the [^{3}H]dopamine uptake operated by LLC-PK1 cells transiently expressing hDAT, hNET, or a chimeric transporter. Transport rates were measured using 5-min incubations, except for the chimeric transporter L (2 min). NaCl or Na^+ contained in the PBS were substituted by twice-equimolar concentrations of sucrose (○) or equimolar concentrations of $Tris^+$ (●), respectively. Concentrations of Na^+ up to 180–300 mM were tested for G and L by addition of NaCl to the medium. Uptake values are expressed as percentages of maximal uptake values, which are presented in Table 1. Data are means ± S.E.M. values from three to four independent experiments performed in duplicate.

another study (M. Syringas, F. Janin, B. Giros, J. Costentin, and J.-J. Bonnet, submitted), current experiments of $Tris^+$ substitution also demonstrate an involvement of the NH_2-terminal part of the transporter in the Na^+-dependence of the uptake. Insertion of cassette I of hNET in hDAT, B and F resulted in significant reductions of Hill number values for A, hNET, and J, respectively.

The introduction of cassette II of hNET in the backbone of hDAT and F gives chimeras G and L, which display a peculiar shape of Na^+-dependence, with an exponential-like increase at low to moderate Na^+ concentrations and a decrease at concentrations above 110 to 150 mM (Fig. 3). This behavior differs from that of either wild-type transporter. This shape is also very different from that of B, so it should not result only from the presence of the second cassette but rather from the insertion of cassette II from hNET origin in a hDAT surroundings. Chimeras A, B, and J, which included cassettes I and II from different origins, did not display such a peculiar shape of Na^+-dependence. Consequently, this shape more probably originates from the presence of cassette II from hNET and the COOH terminal part from hDAT, and more precisely, cassette III from DAT origin. Two findings support this hypothesis. Rather similar shapes of Na^+-dependence were observed for G and L, which differ by their fourth cassette, and the association of cassettes II and III from one parental transporter with cassette IV from the second one produces chimeras F and J, which did not display any peculiar shape of Na^+-dependence.

Finally, present results also support that cassette IV is involved in the Na^+-dependence, because introduction of cassette IV of hNET in hDAT and A decreased the Hill number value in F and J.

As far as the Cl^--dependence is concerned, the exchange of the first cassette gives chimeras A and B, which displayed properties similar to those of the parental transporters (i.e., K_m values near 26–27 mM and a Cl^--independent component of transport for hNET and B, higher K_m values and no Cl^--independent transport for hDAT and A) (Table 1; Fig. 2). This situation differs from that observed in another work, in which the Cl^--dependence of chimeras A and B was intermediate between those of the two parental transporters, suggesting a secondary involvement of this cassette in the Cl^--dependence of the transport (Syringas et al., submitted). However, the ratio of the Cl^- K_m values obtained for parental transporters in the present work (~2) is markedly lower than that found in the other work (~23), rendering moderate differences between parental transporters and chimeras harder to substantiate. Several observations have already shown that properties of the dopamine transport depend on the system used for its study. Various apparent rates of transport were observed in LLC-PK1, C6 glioma, or COS-7 cells expressing hDAT (Gu et al., 1994; Reith et al., 1996; Pifl et al., 1997) and this could be caused by different glycosylations of the transporters in relation with the ability of the cell for trafficking newly synthesized proteins (Patel et al. 1993; Nguyen and Amara, 1996; Patel, 1997). In this respect, it should be kept in mind that all the putative glycosylation sites are borne by the large second extracellular loop, which is present in cassette II. Both human transporters have three glycosylation sites, two at the same locations and one at a divergent site. This cassette II remains of parental origin in chimeras A and B, whereas cassette I is exchanged, and this might explain why these chimeras are more sensitive to the cell line origin in which they are expressed.

The transport activity of G, in which cassette II of hNET was included in the backbone of hDAT, displayed a Cl^- K_m value similar to that of hDAT, suggesting that this cassette is not involved in the Cl^- dependence. In the same way, comparison of chimeras F and J with B and hNET is consistent with a lack of any marked role in the Cl^-- dependence for the second and the third cassette. On the contrary, the involvement of cassette IV in the Cl^- dependence seems quite evident because introduction of cassette IV of hNET in hDAT, A, and G produced chimeras F, J, and L, which share a similar noradrenergic shape of Cl^--dependence with low Cl^- K_m values.

In a putative model of the DAT, Arg 85 and Asn 466 were postulated to constitute a positive site that Cl^- should neutralize before the positively charged dopamine could enter its binding site (Edvarsen and Dahl, 1994). Although further studies are needed to validate this model, it is noteworthy that Arg 85, in the first TMD, is conserved in hNET, whereas Asn 466 in the eighth TMD was changed for Lys in hNET. The resulting increase in positive charge could explain the lower Cl^- K_m for hNET and the role of cassette IV in the Cl^--dependence of the transport.

An overall examination of the present findings demonstrates some striking similarities between domains of the transporters that are involved in the ionic dependence of the uptake and those that play a role in the recognition and the transport of the substrates (Giros et al., 1994; Buck and Amara, 1994, 1995). Thus, the NH_2 quarter-part of the transporter and its COOH-terminal part, and more specifically TMDs 10–11, are involved both in the substrate affinity and/or stereospecificity and in the Na^+- and/or Cl^--dependence of the transport (Fig. 4). Furthermore, the link between cassettes II and III (i.e., between TMDs 3–5 and 6–8), is partly included in the part of the transporter that contains

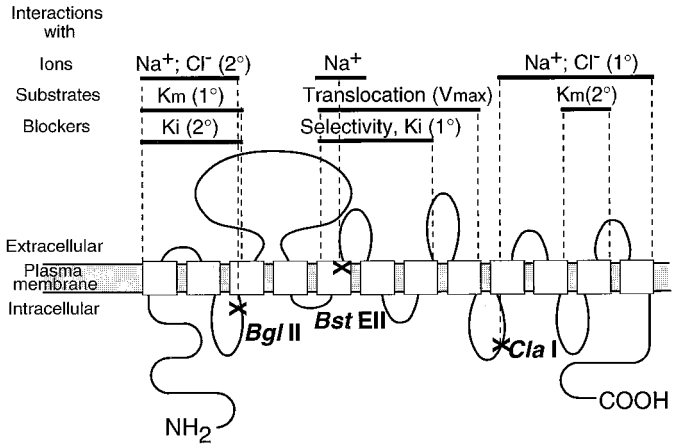

Fig. 4. Summary model of the structural domains of catecholamine transporters influencing uptake of substrates, sensitivity to uptake blockers and dependence to Na^+ and Cl^-. Crosses represent restriction sites (*Bgl*II, *Bst*EII, and *Cla*I) used for generation of the four cassettes. This schematic representation identifies structural domains involved in interactions of DAT and NET with substrates and uptake blockers (Buck and Amara, 1994, 1995; Giros et al., 1994) and with Na^+ and Cl^- (present study). 1° and 2° refer to the primary and secondary involvement of a given region in a function. It is worthy to note that experimental evidence suggesting the involvement of a particular region of the protein in a given function does not mean that other functions are excluded from that region.

important determinants for the translocation activity. This coincidence, demonstrated in the present work, reinforces the idea that Cl^-, and probably Na^+ ions, could occupy binding sites near those of the substrates, essentially for creating charge surroundings favorable to their binding and translocation.

In conclusion, this study has delineated structural domains involved in the ionic dependence of the dopamine transport operated by hDAT and hNET. The Na^+-dependence is demonstrated to depend upon determinants present in three different segments scattered across the transporter, whereas determinants for the Cl^--dependence seem to be mainly located in the COOH-terminal part of the transporter. These results provide useful clues for examining the specific residues that may be involved in the function of transporters, which constitute the biological targets for several important therapeutic agents and drugs of abuse.

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