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The Tyrosine Residue at 1250 of the Insulin-like Growth Factor I Receptor Is Required for Ligand-Mediated Internalization

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The twin tyrosine residues at 1250 and 1251 of the insulin-like growth factor I receptor (IGF-IR) are missing in the corresponding homologous region of the insulin receptor. In this unique region, the tyrosine at 1251 (Y1251) is essential for both transformimg and antiapoptotic activities of the IGF-IR, while Y1250 is dispensable for either of these functions. We show here that a receptor with a mutation at Y1250, but not at Y1251, has lost the ability for ligand-mediated internalization when the mutant receptors are overexpressed in R- cells, derived from a mouse embryo with a targeted disruption of the IGF-IR gene. These results provide evidence that each twin tyrosine at Y1250 and Y1251 of the IGF-IR separately exerts different roles in biologically important signal transductions.

Accumulated evidence indicates that the insulin-like growth factor I receptor (IGF-IR) plays a crucial role not only in cell growth and transformation, but also in the prevention of apoptosis (1-5). Mutational analysis has shown that the tyrosine cluster at 1131, 1135 and 1136 (Y1131, Y1135,Y1136) of the tyrosine kinase domain (6,7) and the tyrosine at 950 (Y950) of the juxtamembrane domain (8) are essential for both mitogenic and transforming abilities of the IGF-IR. On the other hand, the C-terminus, while required for transformation, is dispensable for mitogenicity (9). Within the C-terminus region, the tyrosine at 1251 (Y1251)(10), the histidine at 1293 (H1293) / the lysine at 1294 (K1294)(11) and the serine quartet at 1280-1283 (S1280-1283)(12) are especially important for transforming activity and, to a lesser extent, for protection from apoptosis. O'Connor et al. recently reported that essential domains required for antiapoptotic abilities of the IGF-IR are Y1251 and H1293 / K1294, but not S1280-1283 (13). These amino acids do not exist in the corresponding C-terminus region of the insulin receptor (14). Surprisingly, the antiapoptotic activity is still sustained in the mutant receptors of Y1131, Y1135, Y1136 or the Y950, losing both mitogenic and transforming abilities (13). Thus, it seems likely that mitogenicity and inhibition of apoptosis are essential components of transformation, but they are not sufficient to establish and maintain the transformed phenotype.

Receptor-internalization is a process by which cell surface receptors are rapidly partitioned into intracellular vesicles. It occurs through two different pathways (15) : i) a ligand-dependent, saturable endocytic pathway which is mediated by clathrin-coated pits; ii) a ligand-independent, nonsaturable endocytic pathway which is mediated by smooth pits. The former occurs only when receptors are occupied by ligands and tyrosine kinase activity is required for the internalization of epidermal growth factor (EGF)(16), insulin (17) and IGF-I receptors (18). On the other hand, the latter constitutively occurs irrespective of receptor occupancy (15). Although the roles of the receptor internalization are not fully understood, there is evidence that it serves to attenuate signals by degrading the ligand-receptor complexes transported to lysosomes(19). Indeed, kinase-active, internalization-defective EGF receptors have been reported to enhance morphological transformation and growth activities (16).

In this short communication, we have focused on the role of Y1250 which forms a twin tyrosines site with Y1251; as mentioned above, these two residues are missing in the corresponding site of the insulin receptor. At variance with Y1251, Y1250 is not required for either transformation or antiapoptotic functions (10,13). We show here that Y1250, but not Y1251, is

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required for ligand-mediated internalization using a sensitive method to determine the rate of receptor internalization (15).

MATERIALS AND METHODS

Plasmids. The SK-IGF-IR plasmids including a full coding region of the wild type human IGF-IR cDNA (14) were used as templates to generate mutations by PCR-assisted in vitro mutagenesis as described elsewhere (6-10, 20). The mutated plasmids were excised by SalI and NotI, and ligated to XhoI-NotI sites of pBPV expression vectors (Pharmacia). Mutant plasmids used in the present study were as follows (6-10,20): Y950F (the tyrosine at 950 was replaced by the phenylalanine); K1003A (the lysine at 1003 was replaced by alanine; ATP binding mutant); d1229 (truncated at residue 1229, and thus missing the last 108 aminoacids); Y1250F (the tyrosine at 1250 replaced by phenylalanine); Y1251F (the tyrosine at 1251 replaced by phenylalanine). For selection, pLHL4 (a hygromycin resistance gene)(2,21) or pPDV6+ (a puromycin resistance gene)(10, 22) were used.

Cell lines and cultures. R- cells with a targeted disruption of the IGF-IR genes (1,2, 23) were transfected with expression plasmids including wild type or mutated IGF-IR cDNA, together with those including puromycin or hygromycine resistance genes, and selected by puromycin or hygromycin (2,10). Resultant stable transfectants were used in the present study. Cells were cultured in DMEM supplemented with 5% calf serum and 5% fetal calf serum. Receptor numbers were determined by Scatchard analysis as described previously(10) and ranged from 1 to 9×10^6 receptors per cell.

Determination of specific internalization rate constant (Ke). Specific internalization rate constant (Ke) was determined as described previously (15). The Ke is defined as follows:

$$[LR]_{in} = Ke \cdot \int_0^t [LR]_s^{tot}\, dt$$

where $[LR]_{in}$ and $[LR]_s^{tot}$ represent the concentration of internalized IGF-I and that of the total surface-associated IGF-I, respectively. Thus, plotting the integral of the surface-associated IGF-I from 0 to the time points against internalized IGF-I should give a straight line with a slope of Ke.

Cells (4×10^4 - 2×10^5) grown on 35 mm plastic dishes were switched from growth medium to serum free medium (SFM)(DMEM containing 1 mg/ml BSA and 25 mM HEPES, pH 7.4) at least 4 h before the experiments. The SFM was used for all internalization experiments. The cells were then incubated with 0.5 ng/ml of ^{125}I-IGF-I (Amersham) during incubations lasting up to 5-8 min at 37 °C. After each time of incubations from 0 to the time points with an interval of 1 min, dishes were transferred on ice and washed three times with cold Hank's balanced salt solutions (HBSS). The cell-associated ^{125}I-IGF-I was stripped by incubating cells with cold 0.2 M acetic acid in 0.5 M NaCl solution on ice for 8 min. The radioactivity of the solution including the stripped ^{125}I-IGF-I was measured in an autowell gammma counter. Intracellular radioactivity was determined by measuring that of ^{125}I -IGF-I-stripped cells lysed by 1 N NaOH. Nonspecific binding determined by adding 200 fold excess of cold IGF-I was generally less than 10 %. The data were analyzed by internalization plots, by plotting the integrals of surface binding from 0 to the time points (as a unit of cpm $\times$ min $\times$ plate^{-1}) against the amount of internalized ^{125}I-IGF-I (as a unit of cpm $\times$ plate^{-1}). Integrals were approximated by trapezoidal rule using an interval of Δt=1 min. The specific internalization rate constant (Ke) was determined by the slope obtained from linear regression.

Saturation plots. Cells were incubated with increasing concentrations of ^{125}I-IGF-I ranging from 0.5-16 ng/ml and the Ke was determined at each ^{125}I-IGF-I concentration as described above. In some experiments, incubation time lasted up to 8 min with interval of 2 min, but it did not affect the accuracy. Each Ke value was plotted against the average velocity of ^{125}I -IGF-I uptake between 2 and 8 min at varying ^{125}I-IGF-I concentrations. At steady state, a proper approximation gives the following equation (15):

$$Ke = -Kcp \cdot v + Kcp \cdot Vmax$$

where Ke, Kcp, v and Vmax represent specific internalization rate constant, coated pit constant, velocity of internalization and maximum velocity of internalization, respectively. Since the plot generated by the equation is analogous to a Scatchard plot, values of Vmax and Kcp $\cdot$ Vmax can be obtained from x and y intercepts, respectively, as determined by linear regression. The half saturation occupancy, 1/Kcp, was calculated by the negative reciprocal of the slopes of the regression lines. Analysis was performed for the saturable, high affinity internalization component.

Statistical analysis. Regression analysis was performed by a least squares method and the slopes with 95% confidence limits in internalization plots were presented as Ke for WT and various mutant receptors. For a statistical evaluation of the results, we used the unpaired two tailed Student's t-test or one-way ANOVA followed by Fisher's protected least significant difference (PLSD). A p value <0.05 was considered statistically significant. Data were analyzed using a StatView (Abacus Concepts, inc., Berkley, CA) computer program.

RESULTS AND DISCUSSION

Internalization plots for wild type, Y1250F, Y1251F and other mutant receptors, and each value of specific internalization rate constants (Ke) derived from the slopes of the regression lines are shown in Fig. 1A and 1B. It is obvious that these mutant receptors fall into two distinct categories: normal internalization (group A: WT, Y950F, Y1251F) and abnormal internalization (group B: K1003A, d1229, Y1250F) with remarkably reduced rates (group A vs group B, $p<0.0001$).

It has been reported that the tyrosine kinase activity is essential for ligand-mediated internalization in EGF, insulin and IGF-I receptors (16-18). This was confirmed in our study by the fact that internalization of the kinase-defective mutat K1003A is remarkably inhibited as determined by the method utilized here (Fig.1A,1B). With regard to the juxtamembrane domain, contradictory results were reported in the insulin receptor. Backer et al. (24) and Rajagopalan et al. (25) showed that NPXY and GPLY motifs contained in the domain are important for internalization of the insulin receptor, and the substitution of tyrosines with other amino acids significantly lowered internalization. On the other hand, Kaburagi et al. (26) reported that neither of the motifs are required for internalization and that substitution of tyrosines did not significantly affect internalization. Y950 is contained in the NPXY motif of the IGF-IR, and Prager et al.(27) observed that a mutation of Y950 to alanine significantly lowered internalization. However, Yamasaki et al. reported contrary results (18, 28). Our results favor the latter and the

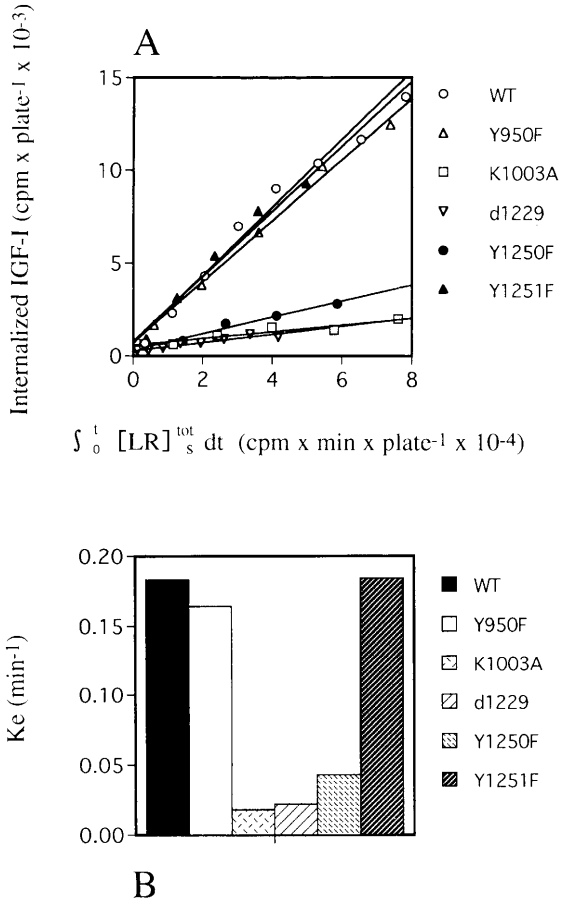

FIG. 1. A: Internalization plots for WT, Y950F, K1003A, d1229, Y1250F, and Y1251F receptors at the concentration of 0.5 ng/ml ^{125}I-IGF-I. Abscissa, integrals of surface binding from 0 to the time points with an interval of 1 min; ordinate, the amount of internalized ^{125}I-IGF-I. Correlation coefficients (r) of the regression lines are as follows: WT, 0.989; Y950F, 0.997; K1003A, 0.951; d1229, 0.948; Y1250F, 0.987; Y1251F, 0.989. Data show a representative result from two to four separate experiments. B: Specific internalization rate constants (Ke) determined by slopes of the regression lines as shown in A. The Ke values with 95% confidence limits are as follows: WT, 0.185 ± 0.029; Y950F, 0.164 ± 0.023; K1003A, 0.018 ± 0.008; d1229, 0.022 ± 0.007; Y1250F, 0.033 ± 0.010; Y1251F, 0.183 ± 0.051.

Y950F mutant receptor was almost normally internalized (Fig.1A,1B). The C-terminus of the EGF receptor contains nine acidic residues which give a strongly negative charge. That region is predicted to be helical and bounded on each end by turns (16). Chen et al. showed that the acidic region is required for internalization of the EGF receptor (16). The insulin and IGF-I receptor also contain an acidic region in the C-terminus, but so far there are no reports concerning the role of this whole region in internalization. We show here that the mutant truncated at residue 1229 (d1229) has an almost similar level of Ke to that of the kinase-deficient mutant receptor (K1003A) (Fig.1A,1B). It seems therefore that the C-terminus is an essential domain for internalization of the IGF-IR. Twin tyrosines (Y1250,1251) locate just before the acidic domain of the IGF-IR and they do not exist in the corresponding homologous region of the insulin receptor. Interestingly, the rate of internalization of Y1250F, but not Y1251F, was significantly lowered. Ke values from four separate experiments for WT, Y1250F and Y1251F were 0.17 ± 0.004, 0.055 ± 0.018 and 0.18 ± 0.013 (min^{-1})(mean ± s.e.), respectively (WT vs Y1250F, $p < 0.001$; WT vs Y1251F, $p > 0.7$; Y1250Y vs Y1251F: $p < 0.001$).

To further characterize the properties of internalization of the Y1250F mutant, saturation plots were performed for WT, Y1250F and Y1251F receptors (Fig.2). As expected, each plot represented two components consisting of saturable, high affinity- and unsaturable, low affinity-internalization pathways (15). It was also clearly shown that the former pathway, namely ligand-mediated internalization is largely affected in the Y1250F receptor. Parameters obtained from the analysis of the saturable pathway were shown in Table 1. Kcp and half saturation receptor occupancy were almost similar among the three receptors and showed 1.1-1.2 $\times 10^{-5}$ and 81,000-91,000 receptors/cell, respectively. On the other hand, Vmax of the Y1250F receptor (4,000 molecules /cell /min) was only 30 % of WT (13,000 molecules/cell/min) or Y1251F (13,000 molecules /cell/min) receptor. One may argue that these results are attributable to clonal variations since only one transfectant was examined. The possibility, however, could be ruled out considering that these transfectants are mixed populations obtained by FACS sorting (10).

The present study clearly demonstrated that the

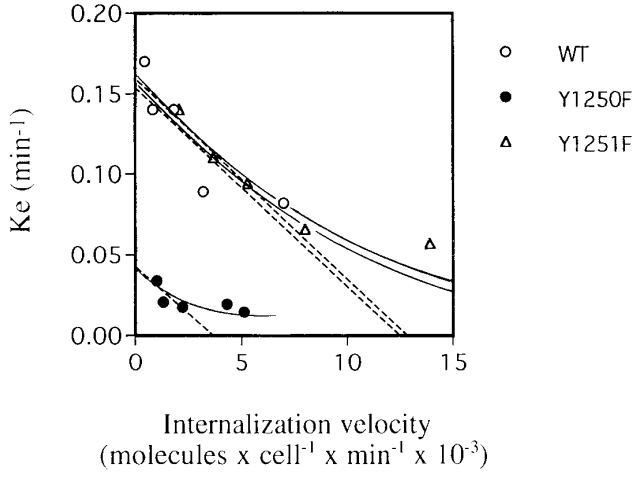

FIG. 2. Saturation plots for WT, Y1250F and Y1251F receptors. Abscissa and ordinate represent internalization velocity and Ke, respectively, as determined at varying concentrations of ^{125}I-IGF-I. Dashed lines represent linear regression lines for the saturable, high affinity internalization component. The equations and correlation coefficients (r) of the regression lines are as follows: WT, $Y = -1.230 \times 10^{-5} X + 0.157$, r=0.871; Y1250F, $Y = -1.101 \times 10^{-5} X + 0.041$, r=0.813; Y1251F, $Y = -1.205 \times 10^{-5} X + 0.160$, r=0.988.

TABLE 1

Internalization Parameters for a Saturable, High Affinity, Internalization Pathway

Cell lines	Vmax molecules/ cell/min $\times 10^{-4}$	Kcp slope $\times 10^{5}$	1/Kcp receptors/ cell $\times 10^{-4}$
WT	1.28	1.23	8.13
Y1250F	0.37	1.10	9.08
Y1251F	1.33	1.21	8.30

Each parameter was determined by linear regressions of the saturable, high affinity internalization component of the saturation plots.

unique tyrosine residue at 1250 of the IGF-IR is required for the ligand-mediated internalization (LMI). The deficiency of the LMI in the Y1250 mutant is not due to the loss of IGF-I-induced tyrosyl phosphorylations like K1003 mutant, because each twin tyrosine mutant showed essentially the same phosphorylation levels of β subunit of the IGF-IR, IRS-1 and Shc compared to those of the wild type as previously reported (10). The exact mechanism existing between Y1250 phosphorylation and LMI of the IGF-IR is not clear at present, however, it is certain that the mechanism is not required for the LMI of the insulin receptor, considering that the tyrosine corresponding to the Y1250 is originally missing in the latter.

Although we could not find correlations between internalization and other functions of the IGF-IR, it was thus revealed that each twin tyrosine at Y1250 and Y1251 of the IGF-IR separately exert different roles in biologically important signal transductions.

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