individuals [12,13]. The primary objective of the present study was to determine the optimum number of home blood pressure measurements in relation to their predictive value for stroke risk. The secondary objective was to compare the predictive value of conventional (screening) and home blood pressure values for the same or less number of measurements (one or two measurements).

Methods

Design

The present report is based on a longitudinal observation of subjects who have been participating in our home blood pressure measurement project in Ohasama, Iwate Prefecture (Japan) since 1987. Ohasama, a rural community, had a total population of 8040 in 1991. The socio-economic and demographic characteristics of this region and the details of the study project have been previously described [11–13]. The study protocol was approved by the Institutional Review Board of Tohoku University School of Medicine and by the Department of Health of the Ohasama Town Government.

Study population

Details of the selection of the study population have been reported previously [12,13]. In brief, of the 1989 eligible individuals aged 40 years and older, more than three measurements (3 days) of home blood pressure data and screening blood pressure data were obtained from 1789 representative individuals [12,13]. Of these, 87 had a previous history of stroke and these subjects were therefore excluded from the present analysis of the relationship between the first onset of stroke and blood pressure values. Of the remaining 1702 subjects, 211 measured their home blood pressure for a period of less than 2 weeks. Therefore, the study population consisted of 1491 individuals. The mean age was 60.6 years and the ratio of men to women was 37 : 63.

Home blood pressure measurements

Physicians and/or public health nurses instructed subjects on how to perform home blood pressure measurements. Subjects were asked to measure their blood pressure every morning within 1 h of waking, in the sitting position after more than 2 min of rest, and to record the results over a period of 4 weeks. The initial 1-day, 2-day, 1-week, 2-week (mean of one, two, seven and 14 measurements, respectively) and multiple (mean of more than 14 measurements; average number of measurements = 25) home blood pressure values were calculated for each individual.

Screening blood pressure measurements

Annual health check-ups, including blood pressure measurements, are available to all Japanese citizens aged 40 years or older. Blood pressure is measured twice consecutively in the sitting position after a rest of at least 2 min by nurses or technicians using a semi-automatic device. The average of the two readings is defined as a screening blood pressure value.

Blood pressure measuring device

Home blood pressure was measured using the HEM 401C (Omron Healthcare Co. Ltd, Kyoto, Japan), a semi-automatic device based on the cuff-oscillometric method [14], which generates a digital display of both systolic blood pressure (SBP) and diastolic blood pressure (DBP).

Screening blood pressure was measured using a USM-700F (UEDA Electronic Works Co. Ltd, Tokyo, Japan), an automatic device based on the Korotkoff sound technique (microphone method).

The average arm circumference for subjects was usually less than 34 cm, so we used a standard arm cuff for both ambulatory and screening blood pressure measurements. Both the home blood pressure measuring device and the screening blood pressure measuring device used in the present study have been previously validated [14,15] and meet the criteria of the Association for the Advancement of Medical Instrumentation [16].

Data analysis

The incidence of stroke and transient ischemic attack (TIA) until 31 December 2001 was investigated through: the Stroke Registration System of the Iwate Prefecture; by tracking death certificates and receipt of National Health Insurance; and by sending questionnaires to each household at the time of home blood pressure measurement. This was then confirmed by checking the medical records of Ohasama Hospital, the only hospital in the town with the facilities for computed tomography and/or magnetic resonance imaging of the brain, where more than 90% of the subjects have their regular check-ups. For 3% of stroke cases, death certificates were the only source of information.

The diagnostic criteria for these subtypes were based on the system for the Classification of Cerebrovascular Disease III by the National Institute of Neurological Disorders and Stroke [17]. The analysis included only the first event for those subjects who had multiple non-fatal events.

Residence in Ohasama as at 31 December 2001 was confirmed by the residents' registration cards. These cards in Japan are accurate and reliable because they are used for pensions and social security benefits. Twenty-seven subjects (1.8%) had moved away and could not be followed up, and 209 deaths (14.0%) were identified from the residents' registration cards.

The association between baseline blood pressure levels

and the incidence of first stroke or TIA was examined using the Cox proportional hazards regression model, which was adjusted for age, sex and smoking status, for the use of antihypertensive medication at baseline, and for history of heart disease, diabetes mellitus or hypercholesterolemia. The dependent variable in these analyses was the number of days from the date of home blood pressure measurement to the date of stroke or TIA, or censoring. Stroke-free and TIA-free survivors as at 31 December 2001 were censored. When examining the incidence of stroke and TIA, we censored cases of death from causes other than fatal stroke events. The mean duration of follow-up was 10.6 years (standard deviation, 2.9; maximum, 13.9 years).

The information on smoking status, on the use of antihypertensive medication at baseline, and on history of heart disease, diabetes mellitus or hypercholesterolemia was obtained from questionnaires sent to each household at the time of home blood pressure measurements and from medical records at Ohasama Hospital. Of the 1491 study subjects, 299 (20%) were classified as current or ex-smokers and 456 (31%) were treated with antihypertensive medication at the baseline, while 15 (1%), 200 (13%) and 189 (13%) subjects were classified as having a history of heart disease, diabetes mellitus or hypercholesterolemia, respectively.

The estimated relative hazard (RH) and the 95% confidence interval (95% CI) of variables were derived from the coefficient and its standard error as determined by the Cox proportional hazards model. Data are shown as mean ± standard deviation. $P < 0.05$ was accepted as indicative of statistical significance. All statistical analyses were conducted using the SAS package (version 8.2; SAS Institute Inc., Cary, North Carolina, USA).

Results

Of the 1491 subjects, 136 had a first onset of stroke or TIA. This was due to cerebral infarction in 95 (69.9%) subjects, intracerebral hemorrhage in 25 (18.4%) subjects, subarachnoid hemorrhage in 10 (7.4%) subjects, TIA in three (2.2%) subjects, and unknown causes in three (2.2%) subjects.

Home and screening blood pressure values

The initial 1-day, 2-day, 1-week, 2-week and multiple home blood pressure values were significantly lower than the screening blood pressure for SBP (Table 1). All home and screening blood pressure values for those patients who developed stroke over the follow-up period were significantly higher than those who did not develop stroke (screening diastolic, $P = 0.02$; other, $P < 0.001$) (Table 1).

Screening blood pressure was significantly correlated with home blood pressure values (SBP: 1-day, $r = 0.45$; 2-day, $r = 0.49$; 1-week, $r = 0.51$; 2-week, $r = 0.52$; multiple, $r = 0.52$; DBP: 1-day, $r = 0.39$; 2-day, $r = 0.43$; 1-week, $r = 0.47$; 2-week, $r = 0.48$; multiple, $r = 0.49$) (all $P < 0.001$), although the correlation coefficient was not very high.

Association between 2-day, multiple home and screening blood pressure values and stroke risk

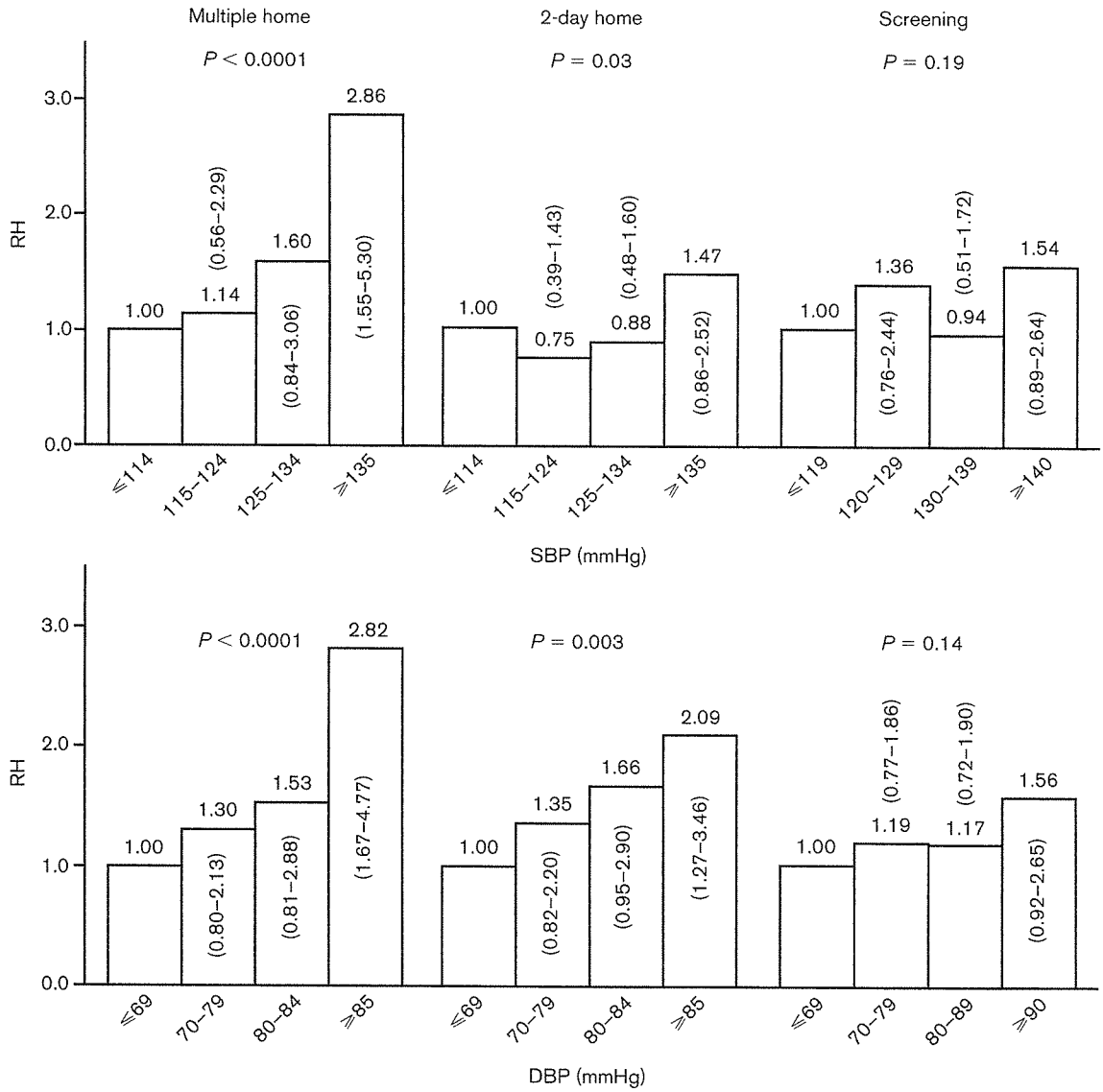
We subdivided the subjects into four groups according to each blood pressure value, and then compared their risk of stroke and TIA (Fig. 1). In the analysis, we treated the lowest group as the reference group. For multiple home SBP measurements, the risk among those with SBP ≥ 135 mmHg (RH = 2.86, $P = 0.0008$) was significantly higher than that among those with SBP < 115 mmHg (reference category), with a significant observable linear trend ($P < 0.0001$). Similarly, multiple home DBP, and 2-day home SBP and DBP values were significantly related to stroke risk (for linear trend, all $P < 0.05$) (Fig. 1). There was a non-significant linear trend between screening SBP, DBP

Table 1 **Blood pressure values of the subjects**

			Stroke and transient ischemic attack	
		Whole	Developed	Not developed
Systolic blood pressure	Screening	132.9 (19.1)	138.9 (19.3)*	132.3 (19.0)
	Home			
	1-day	127.8 (18.6)	137.8 (20.9)*	126.8 (18.0)
	2-day	127.0 (17.1)	136.2 (18.2)*	126.0 (16.7)
	1-week	126.1 (15.8)	135.6 (17.4)*	125.1 (15.3)
	2-week	125.4 (15.4)	135.3 (16.4)*	124.4 (14.9)
	Multiple	125.1 (15.0)	135.2 (16.0)*	124.1 (14.5)
Diastolic blood pressure	Screening	75.5 (11.7)	77.8 (11.1)**	75.2 (11.7)
	Home			
	1-day	76.4 (12.5)	80.5 (13.3)*	76.0 (12.4)
	2-day	75.9 (11.5)	79.6 (11.2)*	75.5 (11.4)
	1-week	75.4 (10.4)	79.2 (10.2)*	75.0 (10.3)
	2-week	75.0 (10.1)	79.1 (10.1)*	74.6 (10.0)
	Multiple	74.8 (9.9)	79.0 (10.1)*	74.4 (9.8)

t test: * $P < 0.001$, ** $P = 0.02$ versus those who did not develop stroke.

Fig. 1



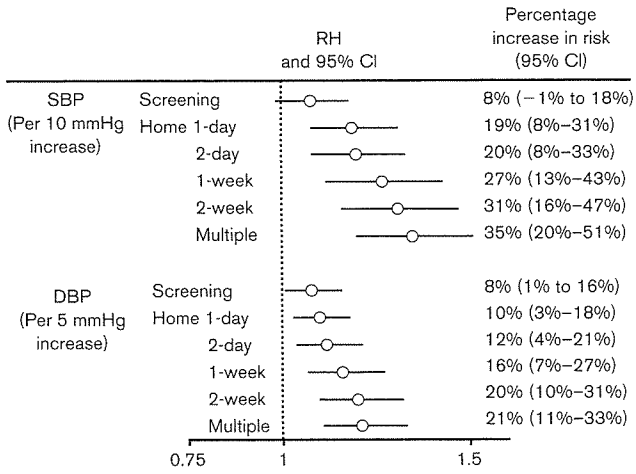
Association between multiple home, 2-day home and screening blood pressure values and stroke risk. Relative hazard (RH) and 95% confidence intervals (CI) of multiple home, 2-day home, and screening systolic blood pressure (SBP) and diastolic blood pressure (DBP) levels adjusted for age, gender, smoking status, the use of antihypertensive medication, history of heart disease, hypercholesterolemia, and diabetes for first symptomatic stroke. *P* for linear trend indicated above the bars. Numbers inside the bars indicate 95% CI. The lowest group was treated as the reference category.

and stroke risk (both $P > 0.1$) (Fig. 1). The initial 1-day, 1-week and 2-week home SBP and DBP values showed a significant linear relationship with stroke risk (all $P < 0.05$) (data not shown).

Predictive values of home and screening blood pressures

As continuous variables, screening blood pressure values showed a linear association with the risk of stroke and TIA (Fig. 2). A 10 mmHg elevation of screening SBP and a 5 mmHg elevation of screening DBP were associated with 8% increases in both the risk of stroke and TIA, respectively (screening SBP $P = 0.07$, screening DBP $P = 0.03$) (Fig. 2). All home blood pressures showed a linear association with the risk of stroke and TIA (Fig. 2). The predictive value of home blood pressure increased progressively with the number of measurements, showing the highest predictive value with multiple home blood pressure measurements: 10 mmHg elevations of 1-day, 2-day, 1-week, 2-week and multiple home SBP values were associated with respective 19, 20, 27, 31 and 35% increases in the risk of stroke and TIA (all $P < 0.01$) (Fig. 2). Similarly, 5 mmHg elevations of 1-day, 2-day, 1-week, 2-week and multiple home DBP values were associated with

Fig. 2



Predictive values of home and screening blood pressures. Relative hazard (RH) and 95% confidence intervals (CI) of 1-day home, 2-day home, 1-week home, 2-week home, multiple home, and screening systolic blood pressure (SBP) and diastolic blood pressure (DBP) levels adjusted for age, gender, smoking status, the use of antihypertensive medication, history of heart disease, hypercholesterolemia, and diabetes for first symptomatic stroke. Open circles are RH expressed as an increase in stroke risk per 10 mmHg elevation of SBP and per 5 mmHg elevation of DBP. Horizontal lines represent 95% CI.

respective 10, 12, 16, 20 and 21% increases in the risk (all $P < 0.01$) (Fig. 2). A similar association was observed for the risk of hemorrhagic stroke (intracerebral and subarachnoid hemorrhage) and for the risk of ischemic stroke (cerebral infarction) (data not shown).

Comparison of predictive values between home and screening blood pressures

When 1-day home and screening blood pressure values were simultaneously included in a Cox model, only 1-day home blood pressure was significantly related with stroke/TIA risk as follows: 1-day home SBP, RH per 10 mmHg elevation = 1.18, $P = 0.002$; screening SBP, RH = 1.02, $P = 0.6$; 1-day home DBP, RH per 5 mmHg elevation = 1.09, $P = 0.03$; screening DBP, RH = 1.05, $P = 0.3$. The model including both 1-day home and screening blood pressure lost 'goodness of fit' when 1-day home blood pressure was removed (SBP likelihood ratio = 9.70, $P < 0.01$; DBP likelihood ratio = 4.91, $P < 0.03$). However, the goodness of fit of the model including both 1-day home and screening blood pressure did not change significantly when screening blood pressure was removed (SBP likelihood ratio = 0.21, $P > 0.5$; DBP likelihood ratio = 1.31, $P > 0.1$). Similarly, the 2-day, 1-week, 2-week and multiple home blood pressure values showed a significantly greater relation with the risk of stroke and TIA than the screening blood pressures (all $P < 0.03$).

Discussion

The present study was based on a longitudinal observation of a representative sample of the general population in a rural Japanese community. Although home blood pressure values showed a linear association with the risk of stroke, the predictive value increased progressively with the number of measurements. There was no threshold for the number of home blood pressure measurements within the range of 1–14 measurements for increasing the predictive power of stroke risk. Goodness of fit of the model including home and screening blood pressures significantly decreased when home blood pressure was removed, but not screening blood pressure. Importantly, even though the number of measurements was the same or less, the 1-day as well as the 2-day home blood pressure values showed a significantly greater relationship with stroke risk than screening blood pressure values.

Home blood pressure is generally self-measured several times over a particular period, but it has not to date been determined how many measurements are needed to provide reliable information in terms of the prognostic significance. Although previous studies of short duration proposed the optimum schedule of home blood pressure measurements as an average of at least 3 days, these studies were based merely on the repeatability and the stability of home blood pressure measurements [18,19]. In this study, multiple home blood pressure measurement (average of 25) was the strongest predictor of stroke or TIA, indicating that the predictive power of home blood pressure was partly dependent upon the number of measurements. We could not determine a threshold for the minimum number of home blood pressure measurements within the range of 1–14 measurements for increasing the predictive power of stroke risk, suggesting that as many measurements as possible, preferably more than 14 measurements, would be recommended for adequate prediction of stroke risk. Initial 1-day home blood pressure was a stronger predictor of stroke or TIA risk than screening blood pressure, even for less number of measurements. These results suggest that, in addition to the number of measurements, other factors such as the lack of the white-coat effect may be associated with superior predictive power. These results also suggest that the view that "the measurement of the initial-single day should be excluded" [19] could not necessarily be applicable from the view point of the prognostic significance.

Previous researchers have reported a stronger correlation of left ventricular hypertrophy determined by electrocardiogram [4] or echocardiography [5,6] with home blood pressure measurements than with conventional measurements. In these studies, home blood pressure was measured for 3 weeks (an average total of more than 20 measurements). In contrast, one study

reported that neither conventional nor home blood pressure was significantly correlated with left ventricular hypertrophy and its treatment-induced regression [20]. In this study, home blood pressure was measured only twice. These results were consistent with our findings that multiple blood pressure measurement at home has a stronger predictive power. It remains to be investigated whether the prognostic value can be improved and a shorter period of home blood pressure measurements may be required if more measurements are obtained on each day.

Compared with those who developed stroke, the difference between screening and home blood pressure (i.e. the magnitude of the white-coat effect) was larger in subjects who did not develop stroke, whose home blood pressure levels were lower than those who developed stroke. These results are consistent with our previous findings that lower home blood pressure levels are associated with larger white-coat effect [21]. These results also suggest that a larger white-coat effect might be a favorable condition for developing stroke.

Although home blood pressure measurement is now widely practised in developed countries, a lack of information on its prognostic significance has partly limited its effective use [22–24]. In this study, we first demonstrated that home blood pressure measurement provides more useful prognostic information for stroke than conventional blood pressure measurement, and that multiple home blood pressure measurements have the strongest predictive power of stroke risk. Although the external validity of the present findings, especially to non-Asian population, would need to be clarified by further studies, in view of these results, we recommend that home blood pressure measurements should be used more effectively in clinical and epidemiological settings for better prediction of individual risk.

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References

1 O'Brien E, Asmar R, Beilin L, Imai Y, Mallion JM, Mancia G, *et al.* European Society of Hypertension Working Group on Blood Pressure Monitoring. European Society of Hypertension recommendations for conventional, ambulatory and home blood pressure measurement. *J Hypertens* 2003; **21**:821–848.

2 Asmar R, Zanchetti A, on behalf on the organizing committee and participants. Guidelines for the use of self-blood pressure monitoring: a summary report of the First International Consensus Conference. *J Hypertens* 2000; **18**:493–508.

3 Imai Y, Otsuka K, Kawano Y, Shimada K, Hayashi H, Tochikubo O, *et al.*, for the Japanese Society of Hypertension. Japanese society of hypertension (JSH) Guidelines for Self-monitoring of Blood Pressure at Home. *Hypertens Res* 2003; **26**:771–782.

4 Ibrahim MM, Tarazi RC, Dustan HP, Gifford RW. Electrocardiogram in evaluation of resistance to antihypertensive therapy. *Arch Intern Med* 1977, **137**:1125–1129.

5 Kleinert HD, Harshfield GA, Pickering TG, Devereux RB, Sullivan PA, Marion RM, *et al.* What is the value of home blood pressure measurement in patients with mild hypertension? *Hypertension* 1984; **6**:574–578.

6 Abe H, Yokouchi M, Nagata S, Ashida T, Yoshimi H, Kawano Y. Relation of office and home blood pressure to left ventricular hypertrophy and performance in patients with hypertension. *High Blood Press* 1992, **1**:279–285.

7 Ezzati M, Lopez AD, Rodgers A, Vander Hoorn S, Murray CJ. Comparative Risk Assessment Collaborating Group. Selected major risk factors and global and regional burden of disease. *Lancet* 2002; **360**: 1347–1360.

8 Asia Pacific Cohort Studies Collaboration. Blood pressure and cardiovascular disease in the Asia Pacific region. *J Hypertens* 2003; **21**: 707–716.

9 Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* 2002; **360**:1903–1913.

10 Eastern Stroke and Coronary Heart Disease Collaborative Research Group. Blood pressure, cholesterol, and stroke in eastern Asia. *Lancet* 1998; **352**:1801–1807.

11 Imai Y, Satoh H, Nagai K, Sakuma M, Sakuma H, Minami N, *et al.* Characteristics of a community-based distribution of home blood pressure in Ohasama in northern Japan. *J Hypertens* 1993; **11**:1441–1449.

12 Tsuji I, Imai Y, Nagai K, Ohkubo T, Minami N, Watanabe N, *et al.* Proposal of reference values for home blood pressure measurement: prognostic criteria based on a prospective observation of the general population in Ohasama, Japan. *Am J Hypertens* 1997, **10**:409–418.

13 Ohkubo T, Imai Y, Tsuji I, Nagai K, Kato J, Kikuchi N, *et al.* Home blood pressure measurement has a stronger predictive power for mortality than does screening blood pressure measurement: a population-based observation in Ohasama, Japan. *J Hypertens* 1998; **16**:971–975.

14 Imai Y, Abe K, Sasaki S, Minami N, Munakata M, Sakuma H, *et al.* Clinical evaluation of semiautomatic and automatic devices for home blood pressure measurement: comparison between cuff-oscillometric and microphone methods. *J Hypertens* 1989; **7**:983–990.

15 Imai Y, Nishiyama A, Sekino M, Aihara A, Kikuya M, Ohkubo T, *et al.* Characteristics of blood pressure measured at home in the morning and in the evening: the Ohasama study. *J Hypertens* 1999; **17**:889–898.

16 Association for the Advancement of Medical Instrumentation. *American National Standards for Electronic or Automated Sphygmomanometers.* Washington, DC: Association for the Advancement of Medical Instrumentation; 1987.

17 National Institute of Neurological Disorders and Stroke Ad Hoc Committee. Classification of cerebrovascular disease III. *Stroke* 1990; **21**: 637–676.

18 Celis H, De Cort P, Fagard R, Thijs L, Staessen JA. For how many days should blood pressure be measured at home in older patients before steady levels are obtained? *J Hum Hypertens* 1997; **11**:673–677.

19 Stergiou GS, Skeva II, Zourbaki AS, Mountokalakis TD. Self-monitoring of blood pressure at home: how many measurements are needed? *J Hypertens* 1998; **16**:725–731.

20 Mancia G, Zanchetti A, Agebiti-Rosei E, Benemio G, Cesaris RD, Fogari R, *et al.*, for the SAMPLE Study Group. Ambulatory blood pressure is superior to clinic blood pressure in predicting treatment-induced regression of left ventricular hypertrophy. *Circulation* 1997; **95**:1464–1470.

21 Hozawa A, Ohkubo T, Nagai K, Kikuya M, Matsubara M, Tsuji I, *et al.* Factors affecting the difference between screening and home blood pressure measurements: the Ohasama Study. *J Hypertens* 2001; **19**: 13–19.

22 Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, *et al.*, National Heart, Lung, and Blood Institute Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, and National High Blood Pressure Education Program Coordinating Committee. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *JAMA* 2003; **289**:2560–2572.

23 Guidelines Committee. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens* 2003; **21**:1011–1053.

24 Japanese Society of Hypertension Guidelines Subcommittee for the Management of Hypertension. Guidelines for the management of hypertension for general practitioners. *Hypertens Res* 2001; **24**:613–634.

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Original Contributions

Prognostic Value of Home Heart Rate for Cardiovascular Mortality in the General Population

The Ohasama Study

Atsushi Hozawa, Takayoshi Ohkubo, Masahiro Kikuya, Takashi Ugajin, Junko Yamaguchi, Kei Asayama, Hirohito Metoki, Kaori Ohmori, Haruhisa Hoshi, Junichiro Hashimoto, Hiroshi Satoh, Ichiro Tsuji, and Yutaka Imai

Background: Recently, the advantages of self-measurement of blood pressure (BP) at home have been recognized. The same advantages could also be applicable to resting heart rate (HR) values assessed at home using a device designed for home BP measurement. However, there have been no studies investigating whether home HR values predict the risk of cardiovascular disease mortality. We therefore investigated the usefulness of HR values in predicting cardiovascular mortality using a device that allowed self-measurement of BP and HR at home.

Methods: The association between the home-measured resting HR and the 10-year risk of cardiovascular mortality was examined in 1780 Japanese individuals ≥40 years of age who had no significant arrhythmias. A Cox proportional hazards model that adjusted for major risk factors was used.

Results: An increase of 5 beats/min in the morning home HR measurement was associated with a 17% increase in the risk of cardiovascular mortality (95% confidence interval 5% to 30%). This relationship was also statistically significant after adjustment for home BP values. Even when home-measured systolic BP was within the normal range (<135 mm Hg), subjects with HR ≥70 beats/min had a higher risk of cardiovascular mortality (relative hazard 2.16, 95% confidence interval 1.21 to 3.85) than those with normal systolic BP and HR values.

Conclusions: Self-measurement of HR at home, together with self-measurement of BP, is a simple method of providing useful clinical information for assessing cardiovascular risk. Am J Hypertens 2004;17:1005–1010

Key Words: Heart rate, blood pressure, cardiovascular disease, mortality, population.

Recently, the usefulness of self-measurement of blood pressure (BP) at home (home BP) has been recognized. It has also been found that multiple measurements lead to a better reproducibility of home BP values.[1,2] This better reproducibility, together with the absence of environmental influences such as the so-called white coat effect[3] and observer bias, diminish regression dilution bias, which results in a better predictive power of home BP measurements having greater predictive power than conventional BP measurements.[4–6] The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation,

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From the Departments of Public Health (AH, KO, IT), Clinical Pharmacology and Therapeutics (TU, JY, KA, HM, YI), Drug Development and Clinical Evaluation (TO, MK, JH), and Environmental Health Science (HS), Tohoku University Graduate School of Medicine and Pharmaceutical Science, Sendai, Japan; and Ohasama Hospital (HH), Iwate, Japan. Tohoku University 21st Century COE Program "Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation" (TO JH HS YI).

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Address correspondence and reprint requests to Dr. Atsushi Hozawa, Department of Public Health, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan; e-mail: hozawa@mail.tains.tohoku.ac.jp

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and Treatment of High Blood Pressure (JNC VII)[7] and the European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension[8] have also emphasized the usefulness of home BP measurements. The same advantages could also be applicable to resting heart rate (HR) values assessed at home (home HR) using a device designed for home BP measurement.

To date, however, there have been no studies investigating whether home HR values predict the risk of cardiovascular disease (CVD) mortality and what value should be made into the reference values. For this reason, we carried out a population-based prospective study to clarify the predictive value of home HR values.

Methods

Design

The present study was a part of a longitudinal observational study of subjects who had been participating in a BP measurement project in Ohasama, Iwate Prefecture, Japan, since 1987.[2–5,9–11] The socioeconomic and demographic characteristics of this region and full details of the project have been described elsewhere.[9,10] The study protocol was approved by the Institutional Review Board of Tohoku University School of Medicine and by the Department of Health of the Ohasama Town Government.

Study Population

The selection of study subjects has been described previously.[11] Briefly, the subjects were ≥40 years of age and were residents of three of the four regions of Ohasama (n = 2716). Hospitalized persons (n = 121) as well as persons who had dementia or who were bedridden (n = 31) were excluded. Individuals who worked out of town (n = 575) were also excluded because the project involved ambulatory BP monitoring. Informed consent to participate in the study was given by 1957 of the 1989 eligible individuals. We have previously confirmed the representativeness of the 1913 subjects who measured their morning home BP on more than three occasions (3 days).[11] For the current analysis, we also excluded 92 more of these subjects from the group of 1913 subjects, because they did not measure their home BP and home HR in both the morning and the evening for at least 3 days. This criterion was based on our previous observation that the average BP value for the first 3 days did not differ significantly from values obtained over the entire study period,[9] and also on the observation that the average home HR value for the first 3 days (morning HR [mean ± SD]: 67.8 ± 8.7 beats/min) did not differ from the value obtained over the entire study period (67.8 ± 7.9 beats/min); indeed, there was a significant correlation between the latter two values (r = 0.87, P = .0001).

We also excluded subjects (n = 41) with a history of significant arrhythmias (such as atrial fibrillation), sick sinus syndrome, or permanent pacemaker implantation. Therefore, the study population comprised of 1780 individuals (mean age, 60.6 years; men:women, 40:60), representing almost 90% of the total eligible population.

Home BP and Heart Rate Measurements

Physicians and public health nurses conducted a health education class to inform the population about home BP and HR recording, taught them how to measure their own BP and HR, and assessed whether the participants were able to measure their own BP correctly. Of the households in the town, 80% attended the class, and public health nurses visited all of the remaining households to provide similar information.[9,10]

The subjects were then asked to measure and record their BP and HR once every morning and evening for 4 weeks. Morning measurements of BP and HR were made within 1 h of awakening, before breakfast or taking any drugs, with the subjects seated and having rested for at least 2 min. Evening measurements of BP and HR were made similarly just before going to bed. Home BP and HR were measured using HEM401C automatic devices (Omron Healthcare Co., Kyoto, Japan), which use the cuff-oscillometric method[12] to generate a digital display of systolic/diastolic BP and HR values. These devices have been validated previously[12] and satisfy the criteria of the Association for the Advancement of Medical Instrumentation.[13] The circumference of the arm was <34 cm in most cases, so we used a standard arm cuff.

The pulse interval was calculated from the pulse wave, which was detected by a manometer incorporated in the equipment. The HR was calculated as follows: HR (beats/min) = 60/average pulse interval. The home BP and HR values for each individual were defined as the means of all measurements obtained for that person.

Follow-Up and Data Collection

Residence in Ohasama Town on December 31, 2001, was confirmed from the residents' registration cards, which were considered accurate and reliable because they are the basis for the payment of pensions and social security benefits in Japan. The underlying cause of any death was determined from the death certificates and classified according to the recommendations of the *International Classification of Diseases, 10th revision* (ICD-10). The primary outcome was mortality from CVD, defined as death from disease of the circulatory system (ICD-10: I00 to I99). Secondary outcomes were mortality from cerebrovascular disease (ICD-10: I60 to I69) or heart disease (ICD-10: I00 to I52 or I70 to I99), respectively.

Information on possible confounding variables (such as smoking status, overweight, use of antihypertensive medication, and history of cardiovascular disease, hypercholesterolemia, or diabetes mellitus) was obtained from questionnaires sent to each subject at the time of starting

Table 1. Characteristics among quintiles of morning home heart rate (HR)

Morning Home HR (beats/min)	≤59	60–64	65–69	70–73	≥74	*P* Value
Number of subjects	371	312	489	282	326	
Age, (ys)	63	61	60	58	59	<.01
Sex (male, %)	51	38	37	35	40	<.01
Smoking status (ever smoker, %)	24	23	22	24	30	.12
Overweight, (BMI >25 kg/m^2), %	34	27	27	34	29	.14
Antihypertensive medication, %	38	32	25	26	29	<.01
Previous history, (%)						
Diabetes	9	13	10	10	12	.42
Hypercholesterolemia	15	13	17	16	17	.48
CVD	8	6	7	7	9	.78
Home BP, (mm Hg)						
Systolic	128	125	124	124	125	<.01
Diastolic	75	75	74	75	77	<.01

BMI = body mass index; BP = blood pressure; CVD = cardiovascular disease.
Continuous variables were tested by ANOVA; categorical variables were tested by χ^2 test.

home BP measurement, from records of annual health check-ups, and from medical records held at the Ohasama Prefectural Hospital.

Statistical Analysis

The association between home HR and BP values and mortality risks were estimated from Cox proportional hazards models[14] adjusted for major CVD risk factors (smoking status, overweight, use of antihypertensive medication, and history of cardiovascular disease, hypercholesterolemia, or diabetes). Subjects who died of other causes were treated as censored. Variables were compared using the χ^2 test or analysis of variance, as appropriate. Differences with a two-tailed P value <.05 were considered statistically significant. All statistical analyses were performed using SAS software, version 8.2 (SAS Institute, Cary, NC).

Results

Home Heart Rate Measurement

The mean home HR values were 67.3 ± 7.8 beats/min for the morning and 69.1 ± 7.9 beats/min for the evening, whereas mean home systolic/diastolic BP values were 125.2 ± 15.1/75.1 ±10.0 mm Hg for the morning and 123.2 ± 14.5/73.4 ± 9.5 mm Hg for the evening. A mean of 23.1 ± 6.9 morning and 23.8 ± 6.9 evening home BP measurements were obtained; similarly, a mean of 22.9±7.0 for morning and 23.6 ± 6.7 evening home HR measurements were recorded. Of the 1780 study subjects, 24% were current or ex-smokers, 30% were overweight (body mass index >25 kg/m^2), and 30% were taking antihypertensive medication. Of the latter, 297 (56%) were receiving calcium antagonists, 107 (20%) were receiving β-blockers, 111 (21%) were receiving diuretics, and 44 (8.2%) were receiving angiotensin-converting enzyme inhibitors. A history of CVD, hypercholesterolemia, or diabetes was recorded in 131 (7%), 283 (16%), and 186 (10%) subjects, respectively.

Table 1 shows the characteristics of the subjects in each quintile, classified on the basis of morning home HR values. The proportion of smokers was higher in the fifth quintile, whereas the values for mean age and mean systolic BP were higher in the first quintile. No significant association was observed between HR values and a history of diabetes, hypercholesterolemia, or cardiovascular disease. The proportion of subjects who were taking antihypertensive medication was higher in the first quintile, although no specific class of antihypertensive drug could account for this difference (data not shown). A similar pattern of characteristics was observed when the quintiles of evening values were examined (data not shown).

Home Heart Rate and Cardiovascular Disease Mortality Risk

The mean duration of follow-up was 10.5 years (maximum 13.9 years). There were 104 CVD deaths (5.8%) and 178 non-CVD deaths (10.0%). Of the 104 CVD deaths, 60 (60%) were due to cerebrovascular disease and 44 (40%) to heart disease. In addition, 35 subjects (2.0%) moved away from the region and were lost to follow-up.

Table 2 shows the relationship between home HR values and the CVD mortality risk. The fifth quintile (≥74 beats/min; relative hazard [RH] = 2.61, P = .008), the fourth quintile (70 to 74 beats/min; RH 2.54, P = .02) of morning HR values were associated with a significantly higher risk of CVD mortality (for linear trend, P < 0.001). When analyzed as a continuous variable, an increase of 5 beats/min in morning home HR as a continuous variable was associated with a 17% increase in the overall CVD mortality risk (95% confidence interval [CI] 5% to 30%, P = .003) after adjustment for major risk factors. Similar tendencies were also observed for the cerebrovascular mortality risk (20% risk increase per increase of 5 beats/min in morn-

Table 2. Relationship between home heart rate (HR) and mortality in all subjects

	No. of Subjects	No. of Events	CVD Mortality RH	95% CI
Morning HR, beats/min				
≤60	371	23	1.12	(0.54–2.33)
61–64	312	11	1.00	
65–69	489	29	1.63	(0.81–3.29)
70–73	282	15	2.54	(1.16–5.58)
≥74	326	26	2.61	(1.29–5.31)
Continuous variable	1780	104	1.17	(1.05–1.30)
Continuous variable*	1780	104	1.17	(1.05–1.30)
Evening HR, beats/min				
≤62	372	17	1.00	
63–66	354	20	1.47	(0.77–2.82)
67–69	270	10	1.14	(0.51–2.52)
70–74	417	30	3.20	(1.73–5.91)
≥75	367	27	2.25	(1.21–4.21)
Continuous variable	1780	104	1.17	(1.05–1.30)
Continuous variable*	1780	104	1.16	(1.04–1.29)

CI = confidence interval; CVD = cardiovascular disease; RH = relative hazards for increase of 5 beats/min in HR.
Relative hazards were calculated by Cox proportional hazard model adjusted for age, sex, smoking status (current *v* ever), overweight, use of antihypertensive medication, history of CVD, diabetes, hypercholesterolemia.
* Further adjustment for systolic blood pressure.

ing HR, 95% CI 4% to 37%, $P = .01$) and the heart disease mortality risk (16%; 95% − 2% to 37%, $P = .09$).

Similarly, evening HR was linearly associated with an increased risk of CVD mortality (17% risk increase per increase of 5 beats/min in HR: 95% CI 5% to 30%, $P = .004$), cerebrovascular mortality (22%; 95% CI 6% to 39%; $P < .01$). However, only weak trends were observed for heart disease mortality (12%; 95% CI −6% to 33%; $P = .21$).

Because we have previously reported that, in this population, home systolic BP had a stronger predictive power for CVD mortality than home diastolic BP,[5] we further examined the effect of home systolic BP on the relationship between home HR and CVD mortality risk. The relationship was essentially unchanged after adjustment for home systolic BP as well as other major risk factors: increases of 5 beats/min in morning and evening HR were associated with 17% (95% CI 5% to 30%, $P < .01$) and 16% (95% CI 4% to 29%, $P < .01$) increases in the CVD mortality risk, respectively.

As the mean age and the proportions of men, smokers, and individuals receiving antihypertensive treatment differed significantly among the quintiles of home HR values (Table 1), we conducted a subgroup analysis based on these characteristics (Table 3). Both morning HR and evening HR values were positively related to the CVD mortality risk without significant interactions with age, sex, smoking status, and antihypertensive treatment.

Home Heart Rate and BP Values and the Risk of Cardiovascular Disease Mortality

Figure 1 indicates the CVD mortality risks among groups defined on the basis of the combined parameters (that is, morning home HR and morning home systolic BP values). We defined a home HR value of ≥70 beats/min as a "high home HR" and a value of <70 beats/min as "normal home HR," given that the fourth and fifth quintiles of both morning and evening home HR values were associated with significantly higher risks than that in the reference category (Table 2). "Systolic home hypertension" was defined according to established reference values of home BP (≥135 mm Hg).[7,8] Hypertensive subjects with a normal HR (RH 1.65; $P = .06$) and normotensive subjects with high HR (RH 2.16, $P < .01$) had significantly higher CVD risk than normotensive subjects with a normal HR (RH 1.00 for reference group). Furthermore, hypertensive subjects with a high HR (RH 3.16, $P < .01$) had the highest CVD risk among the four subgroups. No significant interaction was observed between BP and HR. A similar association was observed among groups defined according to evening values (data not shown).

Discussion

This prospective cohort study demonstrated an independent association between home-measured HR values and CVD mortality in a representative sample of the general population in Japan. On average, each increase of 5 beats/min in home HR was associated with an approximately 17% higher risk of CVD mortality, which was independent of home BP values and other possible confounding factors. This association was also observed for the risks of mortality due to stroke and heart disease. Furthermore, individuals with high home-measured BP and high home-measured HR had a threefold higher risk of CVD mortality than did individuals with normal levels of both variables. These results suggest that self-measured resting HR and

Table 3. Relationship between morning home heart rate (HR) and cardiovascular disease (CVD) mortality by baseline subgroups

	Morning			Evening		
	RH	95% CI	*P* for Interaction	RH	95% CI	*P* for Interaction
Age						
Younger (40–69 y)	1.11	0.94–1.31	.39	1.11	0.94–1.30	.49
Older (≥70 y)	1.20	1.04–1.38		1.19	1.03–1.38	
Sex						
Male	1.16	1.02–1.33	.94	1.13	0.99–1.29	.69
Female	1.13	0.95–1.35		1.16	0.96–1.40	
Smoking status						
Never	1.20	1.04–1.39	.45	1.16	0.99–1.36	.74
Ever	1.12	0.94–1.32		1.13	0.96–1.34	
Antihypertensive medication						
Absent	1.31	1.09–1.57	.09	1.32	1.09–1.60	.06
Present	1.12	0.98–1.28		1.12	0.98–1.28	

Abbreviations as in Table 2

Relative hazards were calculated by Cox proportional hazard model adjusted for age, sex, smoking status, overweight, use of antihypertensive medication, history of CVD, diabetes, hypercholesterolemia and home systolic BP.

BP at home are useful parameters for predicting the CVD risk in the general population.

The present results clearly demonstrated that home HR was an independent predictor of the CVD mortality risk. Home HR measurements are usually taken more frequently under more controlled conditions and with less psychological stress than those obtained in a clinical setting. Several studies[15–21] have reported a positive relationship between clinic HR measurements and CVD mortality after adjustment for BP values, and our results were consistent with those data. However, as we did not measure clinic HR values in the present study population, we could not compare the predictive power of home HR with that of casually measured HR. Nonetheless, in previous studies that treated HR as a continuous variable, an increase of 10 beats/min and an increase of 20 beats/min in clinic HR was reported to be associated with a 23% and a 63% increase in CVD mortality risk,[20,21] respectively, whereas in the present study an increase of 10 beats/min and an increase of 20 beats/min in morning home HR was more strongly associated with CVD mortality risk (37% and 87%, respectively). These results may reflect the possibility that home HR can detect a high risk of CVD mortality more effectively than clinic HR, probably through its better reproducibility resulting from multiple measurements under stable conditions at home.

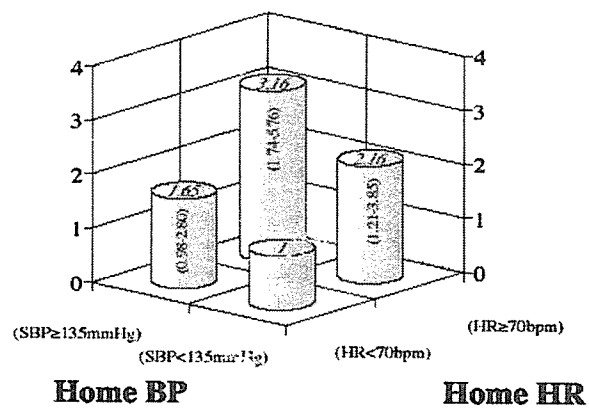

FIG. 1. Home-measured heart rate (HR) and blood pressure (BP) values and the risk of cardiovascular mortality. The relative hazard and 95% confidence interval for cardiovascular mortality associated with each of four groups, defined by a combination of systolic blood pressure (SBP) and HR (in beats/min [bpm]) measured at home in the morning, are shown. The values have been adjusted for age, sex, smoking status, overweight, use of antihypertensive medication, and history of cardiovascular disease, hypercholesterolemia, or diabetes mellitus. **Numbers inside the columns** indicate 95% confidence intervals.

Home-measured HR could be lower than office HR because of the absence of the white coat effect. In fact, some studies that have provided both office HR and home HR values have shown that office HR is higher than home HR.[6,22] Therefore, it is necessary to define the original reference value for home HR. In our study, we found that home HR values ≧70 beats/min were associated with a higher risk of CVD mortality. Therefore, this value could be used as a reference value of home HR to define individuals at high risk when monitoring home HR in this population. However, to generalize these values, further follow-up studies will be needed.

When we estimated the CVD risks among groups defined on the basis of combined parameters, even if home-measured systolic BP was within the normal range (<135 mm Hg), subjects with HR ≥70 beats/min had a higher risk of CVD mortality (RH 2.16, 95% CI 1.21 to 3.85) than those with normal systolic BP and HR values. These results confirmed that a certain proportion of subjects who had higher CVD risk were neglected when HR values were overlooked. Therefore, we consider that the informa-

tion on home HR values should be considered just as important as home BP values.

Our observational study was unable to resolve the issue of whether HR lowering treatments such as β-blocker therapy should be implemented for patients with high HR. The answers to such questions must await large scale, randomized trials of the effects of HR lowering therapy on major causes of morbidity and mortality.

In conclusion, home-measured HR is a strong predictor of the risk of CVD mortality in the general population, and the CVD risk associated with a particular HR value is independent of the home-measured BP value. In this study, a high home HR value was associated with a high risk of CVD, even in normotensive individuals. Moreover, subjects with high home-measured values of both BP and HR showed an extremely high risk of CVD. The HR values obtained at home should be considered just as important as home BP values.

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References

1. James GD, Pickering TG, Yee LS, Harshfield GA, Riva S, Laragh JH: The reproducibility of average ambulatory, home and clinic pressures. Hypertension 1988;11:545–549.
2. Sakuma M, Imai Y, Nagai K, Watanabe N, Sakuma H, Minami N, Satoh H, Abe K: Reproducibility of home blood pressure measurement over a 1-year period. Am J Hypertens 1997;10:798–803.
3. Hozawa A, Ohkubo T, Nagai K, Kikuya M, Matsubara M, Tsuji I, Ito S, Satoh H, Hisamichi S, Imai Y: Factors affecting the difference between screening and home blood pressure measurements: the Ohasama Study. J Hypertens 2001;19:13–19.
4. Ohkubo T, Imai Y, Tsuji I, Nagai K, Kato J, Kikuchi N, Nishiyama A, Aihara A, Sekino M, Kikuya M, Ito S, Satoh H, Hisamichi S: Home blood pressure measurement has a stronger predictive power for mortality than does screening blood pressure measurement: a population-based observation in Ohasama, Japan. J Hypertens 1998; 16:971–975.
5. Hozawa A, Ohkubo T, Nagai K, Kikuya M, Matsubara M, Tsuji I, Ito S, Satoh H, Hisamichi S, Imai Y: Prognosis of isolated systolic and isolated diastolic hypertension as assessed by self-measurement of blood pressure at home: the Ohasama study. Arch Intern Med 2000;160:3301–3306.
6. Bobrie G, Chatellier G, Genes N, Clerson P, Vaur L, Vaisse B, Menard J, Mallion JM: Cardiovascular prognosis of "masked hypertension" detected by blood pressure self-measurement in elderly treated hypertensive patients. JAMA 2004;291:1342–1349.
7. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr, Jones DW, Materson BJ, Oparil S, Wright JT, Jr, Roccella EJ, and National High Blood Pressure Education Program Coordinating Committee: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA 2003;289:2560–2571.
8. European Society of Hypertension-European Society of Cardiology Guidelines Committee: 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. J Hypertens 2003;21:1011–1053.
9. Imai Y, Satoh H, Nagai K, Sakuma M, Sakuma H, Minami N, Munakata M, Hashimoto J, Yamagishi T, Yabe T, Nishiyama A, Abe K: Characteristics of a community based distribution of home blood pressure in Ohasama, a northern part of Japan. J Hypertens 1993;11:1441–1449.
10. Nagai K, Imai Y, Tsuji I, Ohkubo T, Sakuma M, Watanabe N, Kato J, Kikuchi N, Nishiyama A, Sekino M, Itoh O, Satoh H, Hisamichi S, Abe K: Prevalence of hypertension and rate of blood pressure control as assessed by home blood pressure measurements in a rural Japanese community. Clin Exp Hypertens 1996;18:713–728.
11. Tsuji I, Imai Y, Nagai K, Ohkubo T, Watanabe N, Minami N, Itoh O, Bando T, Sakuma M, Fukao A, Satoh H, Hisamichi S, Abe K: Proposal of reference values for home pressure measurement. prognostic criteria based on a prospective observation of the general population in Ohasama, Japan. Am J Hypertens 1997;10:409–418.
12. Imai Y, Abe K, Sasaki S, Minami N, Munakata M, Sakuma H, Hashimoto J, Sekino H, Imai K, Yoshinaga K: Clinical evaluation of semiautomatic and automatic devices for home blood pressure measurement: comparison between cuff-oscillometric and microphone methods. J Hypertens 1989;7:983–990.
13. Association for the Advancement of Medical Instrumentation: American National Standards for Electronic or Automated Sphygmomanometers. AAMI, Washington, DC, 1987.
14. Cox DR: Regression models and life tables. J R Stat Soc [B] 1972;34:187–202.
15. Kannel WB, Kannel C, Paffenbarger RS, Cupples LA: Heart rate and cardiovascular mortality: the Framingham Study. Am Heart J 1987;113:1489–1494.
16. Gillum RF, Makuc DM, Feldman JJ: Pulse rate, coronary heart disease, and death: the NHANES I Epidemiologic Follow-up Study. Am Heart J 1991;121:172–177.
17. Benetos A, Rudnichi A, Thomas F, Safar M, Guize L: Influence of heart rate on mortality in a French population: role of age, gender, and blood pressure. Hypertension 1999;33:44–52.
18. Palatini P, Casiglia E, Julius S, Pessina AC: High heart rate: a risk factor for cardiovascular death in elderly men. Arch Intern Med 1999;159:585–592.
19. Thomas F, Bean K, Provost JC, Guize L, Benetos A: Combined effects of heart rate and pulse pressure on cardiovascular mortality according to age. J Hypertens 2001;19:863–869.
20. Seccareccia F, Pannozzo F, Dima F, Minoprio A, Lo Noce C, Giampaoli S: Heart rate as a predictor of mortality: the MATISS project. Am J Public Health 2001;91:1258–1263.
21. Palatini P, Thijs L, Staessen JA, Fagard RH, Bulpitt CJ, Clement DL, de Leeuw PW, Jaaskivi M, Leonetti G, Nachev C, O'Brien ET, Parati G, Rodicio JL, Roman E, Sarti C, Tuomilehto J, for the Systolic Hypertension in Europe (Syst-Eur) Trial Investigators: Predictive value of clinic and ambulatory heart rate for mortality in elderly subjects with systolic hypertension. Arch Intern Med 2002; 162:2313–2321.
22. Ohkubo T, Hozawa A, Nagatomi R, Fujita K, Sauvaget C, Watanabe Y, Anzai Y, Tamagawa A, Tsuji I, Imai Y, Ohmori H, Hisamichi S: Effects of exercise training on home blood pressure values in older adults: a randomized controlled trial. J Hypertens 2001;19:1045–1052.

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Carboxy Terminus of Glucose Transporter 3 Contains an Apical Membrane Targeting Domain

KOUICHI INUKAI, ANNETTE M. SHEWAN, WENDY S. PASCOE, SHIGEHIRO KATAYAMA, DAVID E. JAMES, AND YOSHITOMO OKA

Fourth Department of Internal Medicine (K.I., S.K.), Saitama Medical School, Saitama 350-0495, Japan; and Division of Molecular Metabolism and Diabetes (Y.O.), Department of Internal Medicine Tohoku University Graduate School of Medicine, Miyagi 980-8574, Japan; Institute for Molecular Bioscience (A.M.S., W.S.P., D.E.J.), University of Queensland, Brisbane 4072, Australia; and Garvan Institute of Medical Research (D.E.J.), St. Vincents Hospital, Sydney 2010, Australia

We previously demonstrated that distinct facilitative glucose transporter isoforms display differential sorting in polarized epithelial cells. In Madin-Darby canine kidney (MDCK) cells, glucose transporter 1 and 2 (GLUT1 and GLUT2) are localized to the basolateral cell surface whereas GLUTs 3 and 5 are targeted to the apical membrane. To explore the molecular mechanisms underlying this asymmetric distribution, we analyzed the targeting of chimeric glucose transporter proteins in MDCK cells. Replacement of the carboxy-terminal cytosolic tail of GLUT1, GLUT2, or GLUT4 with that from GLUT3 resulted in apical targeting. Conversely, a GLUT3 chimera containing the cytosolic carboxy terminus of GLUT2 was sorted to the basolateral membrane. These findings are not attributable to the presence of a basolateral signal in the tails of GLUTs 1, 2, and 4 because the basolateral targeting of GLUT1 was retained in a GLUT1 chimera containing the carboxy terminus of GLUT5. In addition, we were unable to demonstrate the presence of an autonomous basolateral sorting signal in the GLUT1 tail using the low-density lipoprotein receptor as a reporter. By examining the targeting of a series of more defined GLUT1/3 chimeras, we found evidence of an apical targeting signal involving residues 473–484 (DRSGKDGVMEMN) in the carboxy tail. We conclude that the targeting of GLUT3 to the apical cell surface in MDCK cells is regulated by a unique cytosolic sorting motif. (*Molecular Endocrinology* 18: 339–349, 2004)

THE DELIVERY SYSTEM for the targeting of membrane proteins to different cell surfaces in polarized cells has been a subject of considerable interest. Many studies have concentrated on identifying the determinants of basolateral and apical sorting signals at the molecular level (1, 2). A number of basolateral sorting signals described to date have been found to reside in the cytoplasmic domain of membrane proteins (3, 4). Most belong to two classes characterized by either a critical tyrosine-containing motif (YXXØ) (5) or a dileucine or leucine residue adjacent to another bulky hydrophobic amino acid (a.a.) (6, 7). These signals have been demonstrated to associate with adaptor protein 1 (8) and adaptor protein 2 (9, 10), which regulate clathrin assembly at the trans-Golgi network and the plasma membrane, respectively. These signals appear to mediate both efficient delivery to the basolateral membrane and endocytic recycling. Conversely, most of the apical signals that have been characterized to date are found in luminal or transmembrane domains. Although relatively little is known about apical sorting signals, both N-linked (11, 12) and O-linked glycosylation (13, 14) have been shown to play an important role.

Facilitative hexose transporters constitute a family of integral membrane proteins that mediate the transport of sugars across cellular membranes (15). These isoforms share a high level of a.a. sequence homology, and their predicted three-dimensional structure is conserved. Considerable evidence suggests that they contain 12 transmembrane domains with both the N and C termini located on the cytosolic side (15). Despite these similarities, major differences in intracellular trafficking have been noted between individual GLUTs. These differences are best demonstrated in polarized cell types in which different transporters have been localized to discrete surfaces. Glucose transporter 1 and 2 (GLUT1 and GLUT2) are principally found on the basolateral surface in epithelial cells whereas GLUT3 and GLUT5 are mainly targeted to the apical domain (16–19). Similar results are obtained when these transporter isoforms are transfected into Madin-Darby canine kidney (MDCK) cells indicating that this is a universal feature of these proteins that can be recapitulated in a heterologous system (20). These results also demonstrate that MDCK cells provide a useful model for studying the vectorial membrane trafficking of facilitative hexose transporters.

Abbreviations: a.a., Amino acid(s); GLUT, glucose transporter; LDL-R, low-density lipoprotein receptor; MDCK, Madin-Darby canine kidney.

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The asymmetric distribution of GLUTs in polarized cell types has physiological relevance. For example, with respect to the intestinal absorption of fructose, the apically targeted GLUT5 exhibits a high affinity for fructose [low Michaelis-Menten constant (K_m)] (21), whereas the basolaterally targeted GLUT2 exhibits a high V_{max} for fructose (22). Hence, these two facilitative transporters cooperate to achieve efficient absorption of fructose across the gut epithelium.

The molecular mechanisms by which GLUTs are differentially targeted in polarized cells remain to be clarified. Therefore, we attempted to characterize the structural determinants of GLUTs required for this differential targeting. We have expressed a panel of chimeric transporters utilizing various portions of GLUTs 1–5 in MDCK cells and assessed their differential distribution. Our data show that the carboxy-terminal tail of human (h)GLUT3 contains a dominant apical sorting signal that is capable of rerouting both GLUT1 and GLUT2 from the basolateral to the apical cell surface in MDCK cells.

RESULTS

Expression and Analysis of GLUT1/3 Chimeras in MDCK Cells

To clarify the molecular basis for the differential targeting of GLUTs in polarized epithelial cells, we undertook a chimeric strategy whereby different portions of a basolateral transporter and an apical transporter were spliced together and expressed in MDCK cells. Initially, we focused on hGLUT1 and hGLUT3, which are targeted to the basolateral and apical cell surfaces, respectively, in MDCK cells (20). MDCK cells express GLUT1 endogenously but not GLUT3 (20). Initially, we studied the targeting of recombinant hGLUT1 when overexpressed in MDCK cells. Stable cell lines were selected and screened for hGLUT1 expression using a monoclonal antibody that is specific for hGLUT1 (23). This antibody recognizes an epitope in the central loop of hGLUT1 and provides a useful tool for comparing relative expression levels between individual constructs and clones. To verify that this expression system did not result in marked overexpression, we analyzed the glucose transport activities of these clones. In wild-type cells, we observed glucose transport rates of 0.77 ± 0.16 nmol/mg·min and 0.04 ± 0.06 nmol/mg·min across the basolateral and apical membranes, respectively (n = 5, mean ± SD). The glucose transport rates across the basolateral membranes in GLUT1-expressing cell lines were increased at most by 1.5-fold as compared with that observed in wild-type cells. Moreover, we did not observe a significant change in apical transport in clones expressing GLUT1 at this expression level. On the other hand, in clones expressing GLUT3 over a broad range of expression levels, we observed a highly significant increase in transport across the apical membrane (0.56 ± 0.10 nmol/mg·min). Thus, these data provide a good indication that we have performed our studies using nonsaturating expression levels of recombinant transporters. As shown in Fig. 1C (*left upper panel*), at this level of expression the targeting of hGLUT1 was restricted to the basolateral surface as was the case for the endogenous protein. Also shown in Fig. 1C (*right upper panel*) is the distribution of hGLUT3 expressed in MDCK cells. Consistent with our previous findings (20), this protein was highly enriched at the apical cell surface. Whereas these transporters localize to either basolateral or apical membranes, intracellular labeling is also evident. Our focus in this study was the contribution of the cytosolic carboxy terminus to domain-specific cell surface localization and as such we did not characterize the intracellular vesicular compartments through which these chimeras traffic.

The above data provided the basis for our initial studies using hGLUT1/3 chimeras expressed in MDCK cells. We first designed two GLUT1/3 chimeras comprised of different portions of both proteins: hGLUT1/3CT290 contains the N-terminal half of hGLUT1 and the C-terminal half of GLUT3; and hGLUT1/3CT36 is comprised of hGLUT1 in which the carboxy-terminal tail (32 a.a.) has been replaced with that of hGLUT3 (36 a.a.). A scheme of these and other constructs is shown in Fig. 1A. As was the case for all of the constructs described in this study, we selected at least 12 different stable cell lines expressing the recombinant protein of interest and performed detailed analyses on at least three to four different clones for each construct covering a range of expression levels. The targeting of endogenous GLUT1, as well as inulin exclusion, was analyzed in each clone to verify polarity at the time of study (data not shown). Figure 1B shows a Western blot of cell lysates from cells expressing either full-length GLUT3 or the relevant chimeras using an antibody raised against the C-terminal domain of hGLUT3. This antibody did not detect a specific signal in nontransfected MDCK cells, but clearly detected the exogenous hGLUT3 epitope. The chimeras yielded proteins of the appropriate molecular size, *i.e.* similar to that observed for full-length GLUT3. Figure 1C shows the immunolocalization of the hGLUT1/3CT290 (*left lower panel*) and hGLUT1/3CT36 (*right lower panel*) chimeras in MDCK cells as compared with both GLUT1 and GLUT3. Both chimeras were concentrated on the apical domain, similar to the targeting observed for the full-length GLUT3 protein. We also observed labeling of intracellular structures (Fig. 1). This is consistent with our previous studies in which even GLUT1, which is highly concentrated on the basolateral surface (Fig. 1), was also found in intracellular vesicles in MDCK cells (20). These results suggest that the carboxy tails of these transporter proteins determine basolateral *vs.* apical delivery.

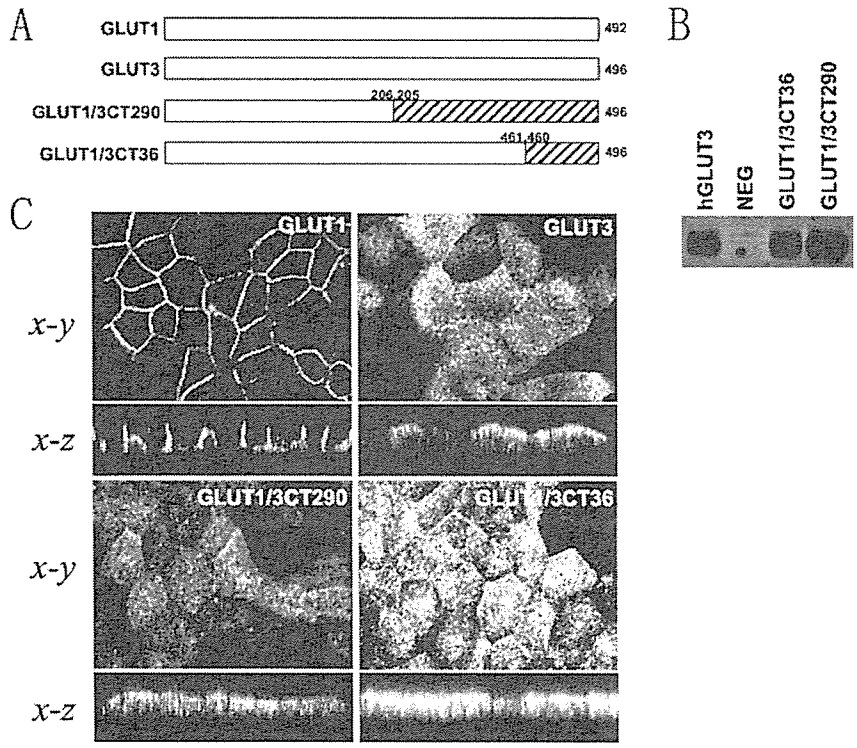

Fig. 1. Immunofluorescence Localizations of GLUT1 and GLUT3 and Their Chimeric GLUTs

A, A diagrammatic representation of the constructs used in these studies is shown. B, Total membrane samples (15 μg) prepared from MDCK cells expressing the indicated proteins were subjected to SDS-PAGE and immunoblotting with an antibody raised against the C-terminal domain of GLUT3. NEG refers to a sample prepared in parallel from parental MDCK cells. Immunoblot data identical to those presented for parental membranes were obtained from nonexpressing but G418-resistant MDCK clones. C, MDCK cells expressing the indicated proteins were plated on glass coverslips, and immunofluorescence was performed as previously described (20). Exogenous transporters were detected using an antibody raised against a short peptide derived from the intracellular loop of hGLUT1 (GLUT1) and an antibody raised against the C-terminal domain of GLUT3 (GLUT3, GLUT1/3CT290, and GLUT1/3CT36). Confocal images were generated using a Zeiss Axiophot fluorescent microscope and a Bio-Rad MRC600 laser scanning head.

Expression of a Low-Density Lipoprotein Receptor (LDL-R)/GLUT1 Chimera in MDCK Cells

It was shown previously that the cytoplasmic tail of the LDL-R possesses two tyrosine-dependent basolateral targeting signals (24, 25). Deletion of these residues resulted in rerouting of the LDL-R to the apical cell surface (24). To determine whether the GLUT1 carboxy tail possesses a basolateral sorting signal, we studied the trafficking of an LDL-R chimera containing the carboxy tail of GLUT1 (LDL-R/G1CT24), in MDCK cells. Several clones expressing the full-length LDL-R and the chimeras were obtained and grown on transwell filters. Trafficking of these proteins was analyzed using a cell surface biotinylation assay (Fig. 2). In agreement with previous studies (24), the wild-type LDL-R was almost entirely targeted to the basolateral membrane, whereas more than 85% of mutated LDL-R (CT37Y-A18) was targeted to the apical membrane. When 24 a.a. from the C terminus of GLUT1 were grafted onto CT37Y-A18, a small portion of the apical proteins relocalized to the basolateral membrane, but the majority remained at the apical membrane (Fig. 2C). These findings suggest that the C-terminal tail of GLUT1 does not contain an autonomous basolateral sorting signal. This does not exclude the possibility of a basolateral sorting signal elsewhere in the GLUT1 molecule. In fact, this seems likely given that it is a multispanning membrane protein with at least three major cytosolic domains. It is also conceivable that sorting domains in these more complex molecules are comprised of discontinuous elements found in discrete domains that interact *in vivo*.

Trafficking of a GLUT1/5 Chimera in MDCK Cells

It was shown previously that GLUT5 is targeted to the apical cell surface in polarized epithelial cells and that apical targeting is regulated via information contained within the central portion of the protein (26). Thus, it is highly unlikely that the C-terminal tail of GLUT5 contains targeting information relevant to trafficking in polarized epithelial cells. Therefore, we reasoned that a GLUT1 chimera containing the C terminus of GLUT5

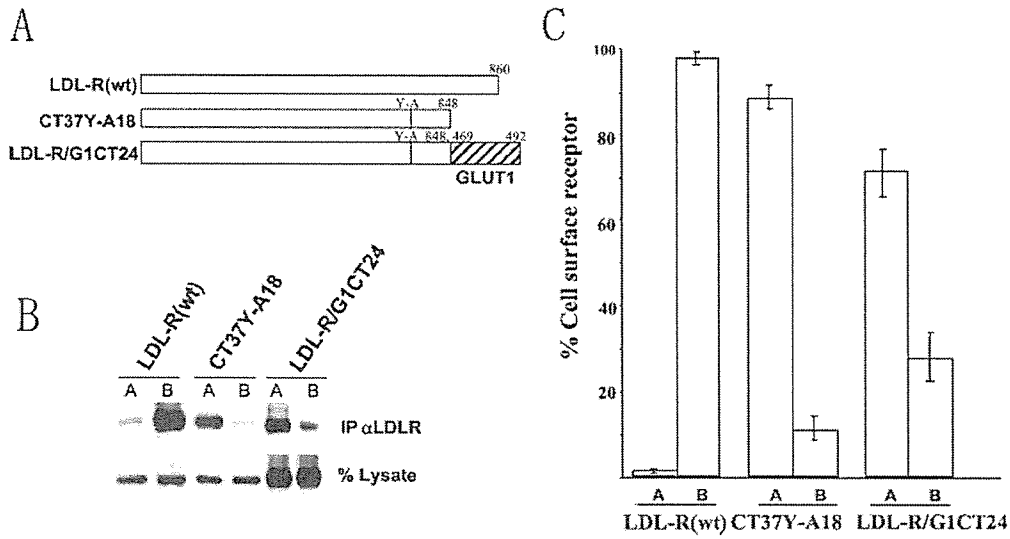

Fig. 2. Cell Surface Distributions of Wild and Mutated LDL-Rs Using Biotinylation Assay

A, A diagrammatic representation of the constructs used in these studies is shown. B, MDCK cells expressing various chimeric proteins were plated onto transwell filters. Apical (A) or basal (B) cell surfaces were incubated in 0.5 mg/ml EZ-link Sulfo-NHS-biotin (Pierce Chemical Co.) for 15 min on ice. Filters were quenched and transferred into lysis buffer, and the solubilized material was cleared by centrifugation. An aliquot of cleared supernatant served as the total expression sample, and the remainder was incubated with streptavidin-agarose to recover biotinylated proteins. Immunoprecipitated proteins were subjected to SDS-PAGE and subsequent Western blot analysis using a polyclonal LDL-R antibody, LB1, with detection performed using goat antirabbit-horseradish peroxidase antibody and enhanced chemiluminescence. C, Values are given as the percent of total cell surface receptors. *Bars* represent the mean ± SD of three independent experiments.

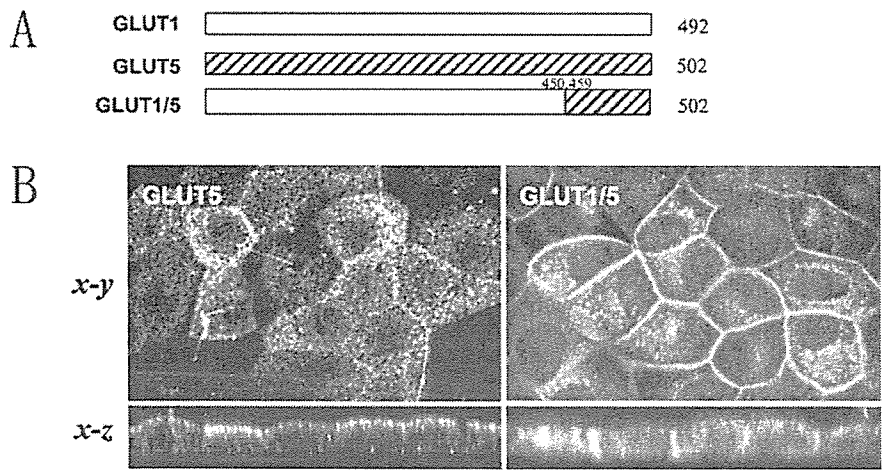

Fig. 3. Immunofluorescence Localizations of GLUT5 and GLUT1/5 Chimeric GLUTs

A, Scheme of the constructs generated for this analysis. B, MDCK cells expressing the indicated proteins were plated onto flamed, glass coverslips and incubated for 5 d to enable tight junction formation and establishment of polarity. Cells were fixed and the domain-specific localizations of exogenous GLUT5 and the GLUT1/5 chimera were assessed by immunofluorescent microscopy using an antibody raised against the C-terminal domain of GLUT5. The GLUT1/5 chimera consists of residues 1–450 of hGLUT1 joined in frame to residues 459–502 of GLUT5.

should provide a useful tool for the present studies. If this chimera targets to the apical cell surface, this will provide definitive proof that the GLUT1 tail contains a basolateral sorting motif. This chimera was expressed in MDCK cells and localized by immunofluorescence microscopy. As shown in Fig. 3, whereas full-length GLUT5 was targeted to the apical surface, the GLUT1/5 chimera was found almost exclusively on the basolateral membrane. In combination with the studies described above, these data strongly indicate that the C-terminal tail of GLUT1 does not contain a basolateral targeting motif.

Expression and Analysis of GLUT2/3 Chimeras in MDCK Cells

To further investigate the apical sorting signal in the C terminus of GLUT3, we undertook an analysis of a panel of chimeras based on hGLUT2 and hGLUT3. Like GLUT1, GLUT2 is targeted to the basolateral cell surface in MDCK cells (20). Initially, we analyzed the targeting of two chimeras, which contained reciprocal portions of GLUT2 and 3. The first, designated GLUT2/3, comprised hGLUT2 up to the end of the last transmembrane domain followed by the cytosolic C-terminal tail of hGLUT3. The second chimera, hGLUT3/2, comprised hGLUT3 up to and including the last transmembrane domain followed by the cytosolic tail of hGLUT2 (Fig. 4A). Both chimeras produced protein products of the correct molecular size when transfected into MDCK cells (data not shown). Confocal immunofluorescence microscopy was used to analyze the targeting of both chimeras in MDCK cells. As shown in Fig. 4B, GLUT2/3 (*left lower panel*) was targeted to the apical membrane and GLUT3/2 (*right lower panel*) to the basolateral membrane. These results support the conclusion that the cytoplasmic carboxy tail of GLUT3 possesses an autonomous apical targeting motif.

Expression and Analysis of GLUT4/3 Chimeras in MDCK Cells

We have previously shown that a chimera composed of the full-length GLUT4 protein and the last 12 a.a. of hGLUT3 appended to the C terminus of GLUT4, when expressed in adipocytes, behaves indistinguishably from GLUT4 (27). This chimera, termed GLUT4/3_{tag}, was expressed in MDCK cells and found to be targeted to intracellular membranes (Fig. 5C, *left panel*) in a manner similar to that previously described for the wild-type GLUT4 protein (20). The intracellular sequestration of GLUT4 is controlled, in part, by an aromatic a.a.-based signal (FQQI) in the cytosolic N terminus of the protein (28). Phenylalanine at position 5 was mutated to alanine in this GLUT4/3_{tag} chimera and expressed in MDCK cells. Localization studies indicated that some GLUT4/3_{tag}F5A was targeted to the basolateral cell surface, whereas others stayed in an intra-

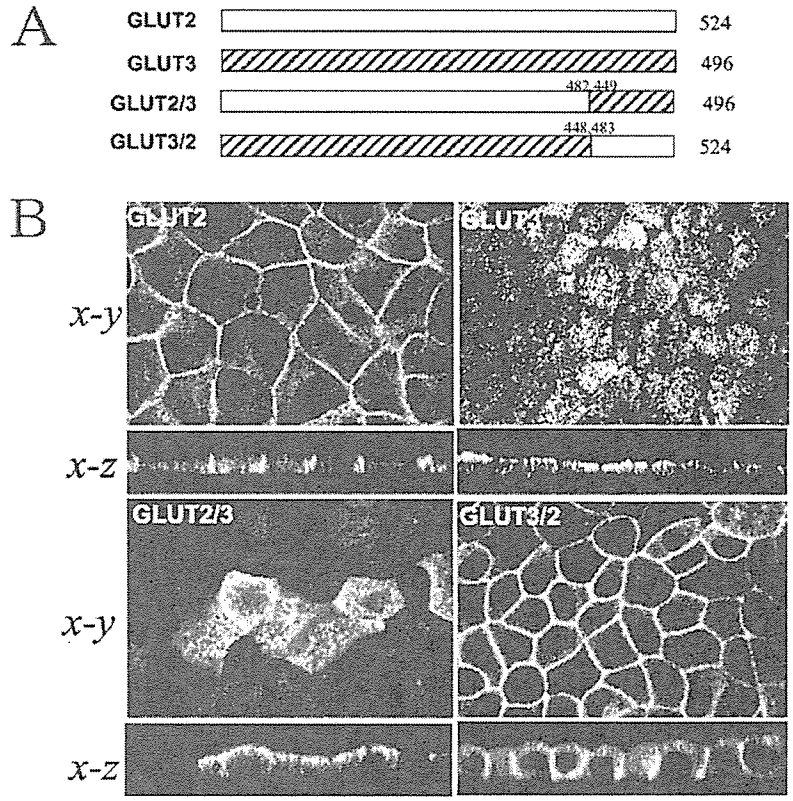

Fig. 4. Immunofluorescence Localizations of GLUT2, GLUT3, and Their Chimeric GLUTs

A, The GLUT2, GLUT3, and the two chimeras generated are represented in this *line drawing*. The chimeras consist of either GLUT2 or 3 up to and including the 12th transmembrane domain of each GLUT with the opposite C-terminal domain fused in frame, generating GLUT2/3 and GLUT3/2. B, At least 4 d before the immunofluorescence study depicted here, MDCK cells expressing the various proteins, as indicated, were plated onto coverslips. The proteins of interest were detected utilizing either an antibody raised against the C-terminal domain of GLUT2 (GLUT2 and GLUT3/2) or an antibody raised against the C-terminal domain of GLUT3 (GLUT3, GLUT2/3). Confocal images were collected and representative images are shown.

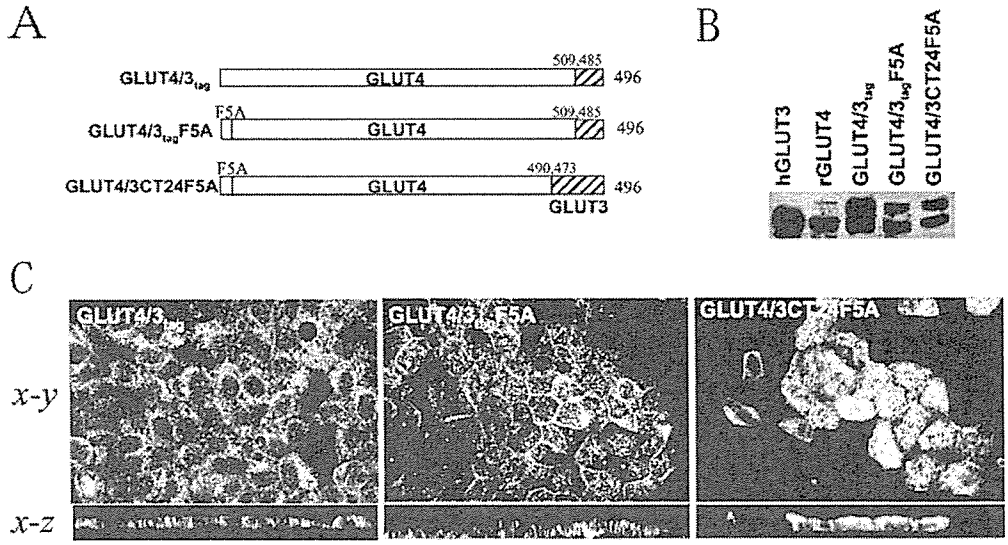

Fig. 5. Immunofluorescence Localizations of GLUT4 and GLUT4/3 Chimeric GLUTs

A, The cDNAs generated for this study and used to transfect MDCK cells are depicted here. As shown, all molecules bear the GLUT3 epitope tag at the extreme carboxy terminus with the Phe5 of GLUT4 mutated to Ala in both GLUT4/3_{tag}F5A and GLUT4/3CT24F5A. B, Western blot analysis of total membrane samples prepared from MDCK cells. Recombinant GLUTs were detected with an anti-GLUT3 antibody. C, MDCK cells expressing GLUT4/3_{tag}, GLUT4/3_{tag}F5A, or GLUT4/3CT24F5A were plated at confluence and allowed to polarize over 5 d. The cellular localization of each of these proteins was assessed by confocal immunofluorescence microscopy using an antibody raised against the GLUT3 carboxy-terminal 12 residues. Representative images are presented.

cellular compartment (Fig. 5C, *middle panel*). Conversely, the chimera GLUT4/3CT24F5A composed of GLUT4 and the cytosolic C-terminal tail of GLUT3 and possessing the Phe5 to Ala mutation was targeted apically (Fig. 5C, *right panel*). Collectively, these data show that the GLUT3 tail possesses an apical targeting signal that does not include the C-terminal 12 a.a.

Further Analysis of GLUT1/3 Chimeras in MDCK Cells

To further define the composition of the apical targeting signal, we examined the targeting of a panel of GLUT1/3 chimeras comprising different portions of the C terminus of GLUT3. Consistent with the data described above (Fig. 5), a chimera in which the last seven a.a. of GLUT1 were replaced with the last 13 a.a. of hGLUT3 (GLUT1/3CT13) was targeted to the basolateral cell surface (Fig. 6C, *middle lower panel*). Intriguingly, addition of a further six a.a. from hGLUT3 (GLUT1/3CT19) resulted in a significant increase in apical targeting although some basolateral targeting was still evident (Fig. 6C, *left lower panel*). However, addition of a further five a.a. from GLUT3 to generate the chimera GLUT1/3CT24 resulted in predominantly apical targeting with little evidence of basolateral localization (Fig. 6C, *right upper panel*). To verify these data, we performed vectorial biotinylation as described above and found that GLUT1/3CT19 was targeted predominantly to the apical side whereas GLUT1/3CT13 was targeted primarily to the basolateral side (Fig. 6D), consistent with our immunofluorescence studies.

As shown in Fig. 6C, expressed GLUT1 occasionally exhibits apparent apical staining in addition to the basolateral staining in immunohistochemical analysis. However, this does reflect, not mixed labeling to different surface domains, but rather both basolateral and intracellular labeling. When the GLUTs reside intracellularly, the outlines of cell nuclei can be seen clearly in x-y images. In contrast, when they localize in apical membranes, the outlines can barely be visualized. When GLUT1s are expressed (Fig. 6C, *left upper panel*), the staining pattern appears to be mixed, similar to those of GLUT1/5 (Fig. 3B) and GLUT3/2 (Fig. 4B). However, this expressed GLUT1 was proved to be located exclusively in the basolateral membrane by biotinylation experiments (Fig. 6D).

DISCUSSION

In the present study, we obtained evidence that the cytosolic carboxy-terminal tail of hGLUT3 contains a targeting motif capable of directing the trafficking of GLUTs 1, 2, and 4 to the apical cell surface in MDCK cells. By examining the targeting of a series of more defined GLUT1/3 chimeras, we found evidence for the presence of an apical targeting signal involving residues 473–484 (DRSGKDGVMEMN) in the carboxy tail. The finding of special interest is that chimeras con-

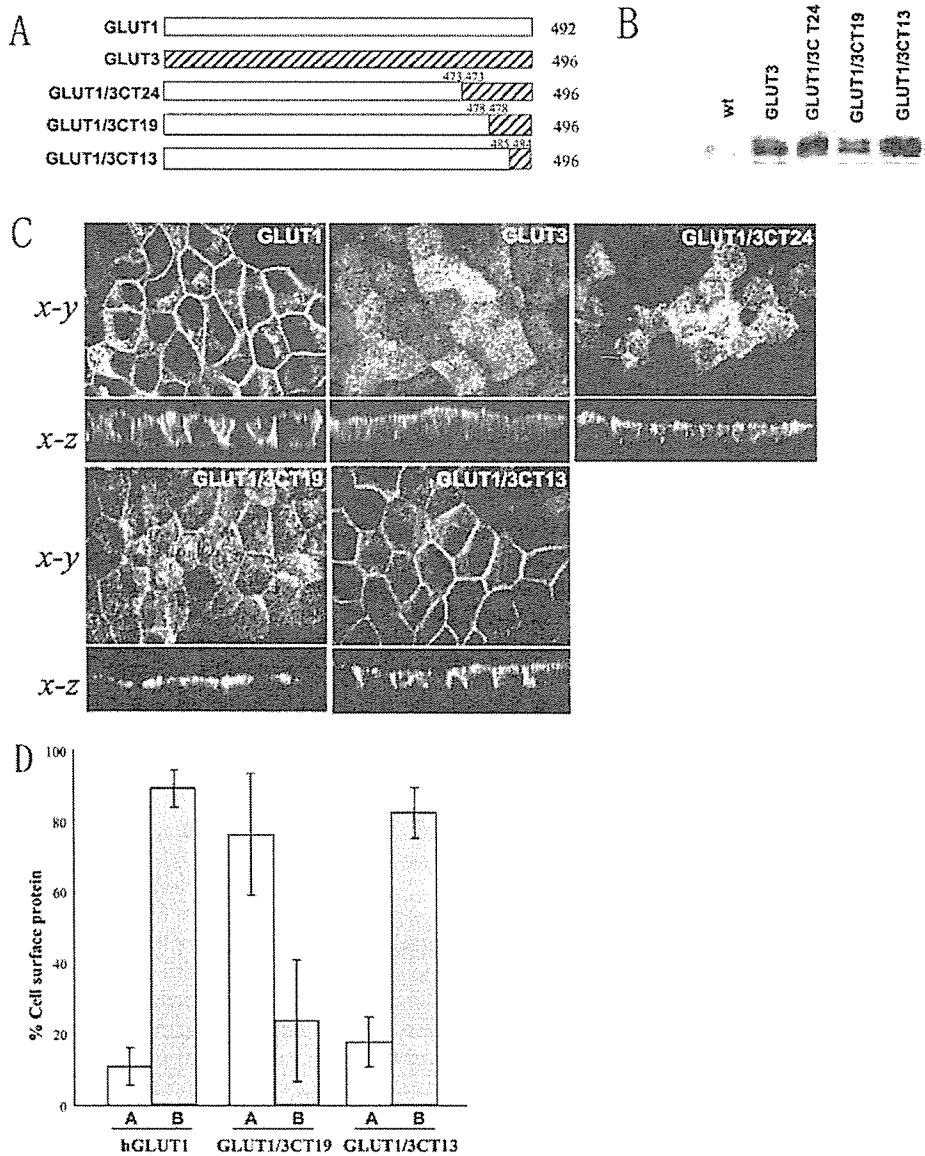

Fig. 6. Immunofluorescence Localizations of GLUT1, GLUT3, and Their Chimeric GLUTs

A, Stable MDCK cell lines were generated by transfection with cDNA, as depicted, and selection in G418. GLUT1/3CT24 consists of a.a. 1–473 of GLUT1 and a.a. 473–496 of GLUT3. Similarly, GLUT1/3CT19 and GLUT1/3CT13 were constructed by fusing a.a. 1–478 (CT19) or a.a. 1–485 (CT13) of GLUT1 to a.a. 478–496 (CT19) or a.a. 484–496 (CT13) of GLUT3. B, Membrane samples were prepared and subjected to SDS-PAGE and Western blot analysis to assess expression levels of GLUTs in MDCK cells. Membranes were incubated with an antibody raised against the C-terminal domain of GLUT3. C, MDCK cells were transfected (with cDNA as indicated in panel A) and clones were analyzed by immunofluorescence microscopy to ascertain the domain-specific localization of the recombinant transporters. Cells were fixed, permeabilized, and immunolabeled using an antibody raised against a short peptide derived from the intracellular loop of hGLUT1 (GLUT1) and an antibody raised against the C-terminal domain of GLUT3 (GLUT3, GLUT1/3CT24, GLUT1/3CT19, and GLUT1/3CT13). Confocal images were generated using a Zeiss Axiophot fluorescent microscope and a Bio-Rad MRC600 laser scanning head. D, Cell surface localizations of overexpressed hGLUT1, hGLUT1/3CT19, and hGLUT1/3CT13 were assessed using a domain-specific biotinylation assay. Cells were grown at confluence for 4–5 d on Transwell filters. Apical and basolateral cell surface proteins were biotinylated by addition of 0.5 mg/ml EZ-Link-Sulfo-NHS-Biotin (Pierce Chemical Co.) for 15 min. Biotinylated proteins were recovered and subjected to SDS-PAGE followed by Western blotting with antibodies specific for hGLUT1. Chemiluminescent bands were quantified using a Bio-Rad 600 densitometer, and signals detected were corrected to account for loading differences.

taining the last 12 or 13 a.a. of GLUT3 did not exhibit significant apical targeting, whereas a chimera containing a further seven a.a. from GLUT3 (GLUT1/3CT19) exhibited significant apical targeting. Thus, fine mapping of the C-terminal tail of hGLUT3 has narrowed the apical sorting information to a small

region encompassing residues DGVMEMN, which corresponds to residues PAGVELN in rodent GLUT3. It is noteworthy that this chimera (GLUT1/3CT19) was partially targeted to the basolateral membrane whereas GLUT1/3CT24, which contained an additional five a.a. from GLUT3, was targeted almost indistinguishably from wild-type GLUT3. To our knowledge, no similar targeting motifs have been reported in other apical membrane proteins, and we have been unable to identify a similar motif in other apical membrane proteins. Most noteworthy is the presence of two conserved hydrophobic residues surrounding an acidic residue in both the rodent (VEL) and human (MEM) sequences. Future studies will be aimed at identifying the roles of these residues.

Based upon our initial studies with GLUT1/3 chimeras (Fig. 1), we hypothesized that the GLUT1 tail contained a basolateral signal that was disrupted in this chimera, resulting in default trafficking to the apical cell surface. However, detailed studies showed that this was not the case and argued in favor of an apical signal in GLUT3. Most notably, the GLUT3 tail facilitated apical targeting when grafted onto GLUT1, 2, or 4, all of which normally recycle via the basolateral cell surface. Thus far, two separate classes of basolateral sorting signals have been identified. These are characterized by either an essential tyrosine residue, or a dileucine motif (5–7). Similar kinds of motifs could not be found in the C termini of either GLUT1 or GLUT2 (Fig. 7). The LGA residues are conserved between GLUT1 and GLUT2 in the C terminus. However, mutating all of these residues did not disrupt the basolateral targeting of GLUT1 (data not shown). We also prepared a GLUT1 mutant missing the last three a.a. because this domain has been shown to act as a binding site for PDZ domain-containing proteins (29, 30). However, the distribution of this truncated GLUT1 was exactly the same as that of wild-type GLUT1 (data not shown). Thus, using this type of approach, we were unable to identify a basolateral targeting domain in the tail of GLUT1. Consistent with this conclusion, the GLUT1 tail did not significantly alter the apical targeting of a tail minus LDL-R, a construct that has been successfully used to map basolateral sorting signals in cytosolic tails (Fig. 2). Moreover, the cytosolic tail of GLUT5, which does not contain a basolateral sorting signal, did not disrupt the targeting of GLUT1 (Fig. 3). Taken together, these studies provide compelling evidence that the C-terminal tail of GLUT3 contains an apical sorting signal. This signal is obviously very dominant, being able to overcome basolateral sorting signals in GLUTs 1, 2, and 4.

It was reported previously that some apical targeting motifs function by selectively partitioning into lipid raft domains in the Golgi (31). Intriguingly, it was reported recently that, in nonpolarized epithelial cells, whereas a substantial portion of GLUT1 was found in detergent-resistant membrane domains, this was not the case for GLUT3 (32). Hence, these data support the notion that GLUT3 is sorted via a non-lipid raft-mediated domain; this is consistent with the present data indicating such a domain to be located in the cytosol, perhaps allowing the transporter to interact with cytosolic targeting machinery. Several other reports have recently found narrowly defined sequences or short peptides in the C-terminal cytoplasmic tails of membrane proteins to have a role in apical targeting (33–37). Among them, multiple autonomous signals for apical targeting in the same protein have been reported (33, 34). These signals include PDZ-interacting domains (35) and β-turn structures (36) as well as less well-defined signals. Taken together, these observations suggest the apical sorting mechanism defined by cytosolic sorting signals to be less well defined than luminal or transmembrane signals, and further work is required to determine whether there is some type of homology among these motifs.

GLUT3 has been shown to undergo specialized targeting in other cell types. It is targeted to the limited membrane of secretory granules in platelets (38) and to the tail of sperm cells (39). Moreover, there is evidence that GLUT3 is targeted to axons in neurons (our unpublished data) and also to neuronal vesicles (40). It will be intriguing to determine whether the cytosolic C terminus is involved in each of these unique trafficking processes, which would imply a common mechanism. Consistent with this, it was previously suggested that similar rules govern the targeting of membrane proteins in epithelial cells and neurons (41).

The identification of this apical targeting signal in the GLUT3 tail may have significant utility in studying the structure/function of sugar transporters in mammalian

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human GLUT1-KVPETKGRTFDEIASGFRQGGASQSDKTPEEIFHPLGADSQV        492
human GLUT2-KVPETKGKSFEEIAAEFQKKSGSAHRPKAAVEMKFLGATETV        524
mouse GLUT2-KVPETKGKSFEEIAAEFRKKSGSAPPRKAAVQMEFLASSESV        523
human GLUT3-KVPETRGRTFEDITRAFEGQAHGADRSGKD--GVMEMNSIEPAKETTTNV     496
mouse GLUT3-KVPETKGRTFEDIARAFEGQAH-----SGKGPAGV-ELNSMQPVKETPGNA    493
rat   GLUT3-KVPETKGRTFEDITRAFEGQAH-----SGKGSAGV-ELNSMQPVKETPGNA    493
human GLUT4-RVPETRGRTFDQISAAFHRTPSLLEQEVKPSTELEYLGPDEND        509
human GLUT5-IVPETKAKTFIEINQIFTKMNKVSEVYPEKEELKEL---PPVTSEQ     501
rat   GLUT5-VVPETKGRTFVEINQIFAKKNKVSDVYPEKEE-KELNDLPPATREQ     502
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Fig. 7. Comparison of the Cytoplasmic Tails of Human and Rodent GLUTs

The putative a.a. sequence, which is suggested to have an apical sorting signal, is *underlined*.

cells. A major complication of studying transport kinetics of GLUTs after their expression in mammalian cells is that there is always a very high background due to the presence of endogenous transporters. We previously found that glucose transport across the apical cell surface in MDCK cells is negligible because most of the uptake occurs via the basolateral surface (20). By transplanting the apical signal from GLUT3 onto other transporters it should be feasible to redirect them to the apical surface, as shown here, which should afford ideal conditions for studying transport kinetics in a more appropriate environment.

In conclusion, we have analyzed the asymmetric distribution of facilitative GLUTs by expressing chimeric transporters utilizing various portions of GLUTs (GLUT1–GLUT5) in MDCK cells. Our present data strongly suggest that the apical sorting signal resides in the C-terminal tail of GLUT3.

MATERIALS AND METHODS

cDNA Constructs

Chimeric cDNAs were produced according to previously described methods (42), which allowed us to swap different domains from different transporter isoforms at any desired junction. A hGLUT1 cDNA (23), a hGLUT2 cDNA (42), a hGLUT3 cDNA (27), a hGLUT4 cDNA (28), and a rat GLUT5 cDNA (43) were used as PCR templates. Cytomegalovirus-based expression plasmid pCB6 was generously supplied by Dr. Mellman (4). Fragments prepared by PCR were fully sequenced and observed to have no unexpected mutations. cDNAs encoding wild-type and chimeric GLUTs were ligated into pCB6 vectors.

Cell Culture and Transfection

MDCK cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C, in 5% CO_2. Lipofectamine reagent, Opti-MEM I, and G-418 (geneticin) were purchased from GIBCO Life Technologies (Eggenstein, Germany). One day before transfection, MDCK cells were trypsinized and seeded onto a 60-mm plastic culture dish at 6 x 10^5cells per dish. The following day, transfection procedures were performed using 30 μl lipofectamine diluted in 300 μl Opti-MEM I (supplemented with 20 mM L-glutamine) and 6 μg GLUT/pCB6 diluted in 300 μl of supplemental Opti-MEM-I/60-mm dish. Cells were incubated in the presence of the lipofectamine-DNA mix for 5 h at 37 C, in 5% CO_2, and then incubated overnight in DMEM-10% fetal calf serum-L-glutamine. Forty-eight hours after transfection, each transfected 60-mm dish was split into three 150-mm dishes and incubated with G-418 (0.8 mg/ml). Colonies resistant to G-418 were isolated after 10–14 d and screened for protein expression by Western blotting.

Western Blotting

To screen for positive expression of the chimera of interest, membrane protein samples (20 μg protein) were subjected to SDS-PAGE employing a 10% resolving gel. Proteins were transferred to a nitrocellulose membrane. Membranes were incubated for 1 h at 37 C with the appropriate primary antibody. After three 10-min washes in PBS-0.1% Tween 20, the membranes were incubated at room temperature for 1 h with a horseradish peroxidase-labeled donkey antirabbit secondary antibody (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) diluted 1:10,000 in PBS-0.2% BSA. After three further washes, labeled proteins were visualized using the enhanced chemiluminescence detection method (Amersham Life Science).

Preparation of Total Membrane Fractions

Total membrane fractions were prepared from MDCK cells after homogenization in 20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose buffer containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 250 μM phenylmethylsulfonylfluoride) and centrifugation at 50,000 rpm in a Beckman TLA100–3 rotor (Beckman Coulter, Inc., Fullerton, CA) for 60 min. The membrane pellet was resuspended in 20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose buffer and stored at −80 C before use.

Protein Assay

The protein concentration of total membrane fractions was determined using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Immunofluorescence Microscopy

Cells were plated at near-confluent density on glass coverslips and fixed 5 d later in 2% paraformaldehyde-PBS for 1 h at room temperature. Polarization was indicated by the presence of domes or blisters in the cultures. Coverslips were washed three times in PBS, and then quenched for 15 min in 0.2% Triton X-100. After an additional three washes in PBS, coverslips were blocked for 30 min in 2% horse serum-PBS and then washed twice in PBS. Primary antibodies were diluted in 0.1% horse serum-PBS, and incubations were carried out at 4 C overnight. Fluorescein isothiocyanate-conjugated antirabbit Ig secondary antibody diluted in 0.1% horse serum-PBS was applied after three 5-min washes in PBS. After a 1-h incubation, at room temperature, coverslips were washed three times in PBS for 5 min each and then mounted in 1% propyl gallate-50% glycerol-PBS. Confocal images were generated with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) and a Bio-Rad MRC600 confocal laser scanning head (Bio-Rad Laboratories, Inc., Hercules, CA). At least one G-418-resistant clone, which was negative by immunoblotting, served as a negative control for immunofluorescence microscopy.

Cell Surface Biotinylation

Localization of the LDL-R constructs and GLUT1/3 chimeras was assessed utilizing a domain-specific biotinylation assay. Briefly, MDCK cells expressing various chimeric proteins were plated onto transwell filters (Corning, Inc., Corning, NY) and grown for at least 4 d at 37 C and 5% CO_2. To assess the integrity of the monolayer, growth media containing [^{14}C]inulin (0.1 mCi/ml at 1:2000 dilution) were added to the apical chamber and incubated for 1 h. After this incubation, aliquots of medium from both the apical and basal chambers were counted (Coulter Counter, BD, Beckman Coulter, Inc.). Transwells with less than 0.5% transport were used in experiments. Filters were washed three times with ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS+). Apical or basal cell surfaces were incubated with 0.5 mg/ml EZ-link Sulfo-NHS-biotin (Pierce Chemical Co.) for 15 min on ice. This process was repeated, and the free biotin reagent was then quenched by washing with PBS+ containing 50 mM