The thiamin transporter encoded by SLC19A2 and the reduced folate carrier (RFC1) share 40% homology at the protein level, but the thiamin transporter does not mediate transport of folates. By using murine leukemia cell lines that express no, normal, or high levels of RFC1, we demonstrate that RFC1 does not mediate thiamin influx. However, high level RFC1 expression substantially reduced accumulation of the active thiamin coenzyme, thiamin pyrophosphate (TPP). This decreased level of TPP, synthesized intracellularly from imported thiamin, resulted from RFC1-mediated efflux of TPP. This conclusion was supported by the following observations. (i) Efflux of intracellular TPP was increased in cells with high expression of RFC1. (ii) Methotrexate inhibits TPP influx. (iii) TPP competitively inhibits methotrexate influx. (iv) Loading cells, which overexpress RFC1 to high levels of methotrexate to inhibit competitively RFC1-mediated TPP efflux, augment TPP accumulation. (v) There was an inverse correlation between thiamin accumulation and RFC1 activity in cells grown at a physiological concentration of thiamin. The modulation of thiamin accumulation by RFC1 in murine leukemia cells suggests that this carrier may play a role in thiamin homeostasis and could serve as a modifying factor in thiamin nutritional deficiency as well as when the high affinity thiamin transporter is mutated.

The reduced folate carrier (RFC1), first cloned in 1994, mediates transport of reduced folates critical to one carbon-requiring biosynthetic reactions in mammalian cells and is a member of the major facilitator superfamily of transporters (1–3). RFC1 also delivers MTX and new generation antifolates into a variety of tumors, particularly those of hematopoietic origin. (4) RFC1 exchanges folates with a broad spectrum of inorganic and organic anions, and high extracellular concentrations of a variety of organic phosphates competitively inhibit RFC1-mediated folate influx (5–7). This interaction between RFC1 and organic phosphates results in the uphill transport of folates into cells linked to the organic phosphate gradient across cell membranes (5).

Structurally unrelated to the folates, thiamin plays an essential role in glycolysis and oxidative decarboxylation reactions after conversion to the coenzyme thiamin pyrophosphate by thiamin pyrophosphokinase in cells. Thiamin is also transported across cell membranes by a carrier-mediated process (8). Thiamin deficiency, reflected in a decrease in plasma thiamin concentration and TPP levell levels in erythrocytes, results in a variety of clinical abnormalities including cardiovascular and neurological disorders (9). Thiamin deficiency due to impaired transport results in the thiamin-responsive megaloblastic anemia syndrome, a disorder also associated with deafness and diabetes mellitus (10, 11). Positional cloning with families inheriting this autosomal recessive disease led to the recent identification of the thiamin transporter gene SLC19A2 (12–14).

The thiamin transporter encoded by SLC19A2 is highly homologous to RFC1, sharing an amino acid identity of 49% and similarity of 55%, and both are predicted to have 12 transmembrane domains. Despite the similarity between these two proteins, the thiamin transporter, when expressed in HeLa cells, was not found to transport folates (15). In the current report, the impact of RFC1 function on thiamin transport and accumulation of its active coenzyme metabolites was studied in murine leukemia cells. Although RFC1 was not found to transport thiamin, it does transport phosphorylated thiamin derivatives, thereby modulating the intracellular accumulation of active thiamin metabolites.

MATERIALS AND METHODS

Chemicals—[3,5,7-3H]MTX (5.7 Ci/mmol) and [3H]thiamin hydrochloride (20 Ci/mmol) were obtained from Amersham Pharmacia Biotech, and [3H]TPP (generally labeled, 4.2 Ci/mmol) was custom-made by Moravek Biochemicals (Brea, CA). Unlabeled MTX was provided by Lederle Laboratories (Carolina, Puerto Rico), and thiamin, TMP, and TPP were purchased from Sigma. Tritiated MTX was purified by high performance liquid chromatography before use (16), and tritiated thiamin and TPP were used directly after purity was confirmed by HPLC.

Cells Lines and Culture Conditions—G1a, G2, D10, and MTX rA cells were selected from murine leukemia L1210 cells in the presence of MTX, with or without chemical mutagenesis, as previously reported (17–21). All four cell lines harbor mutations in RFC1 resulting in impaired or absent transport mediated by RFC1. Both R16 and T2 are cell lines with high level expression of RFC1, obtained by transfecting murine RFC1 cDNA into MTX rA or wild-type L1210 cells, respectively (21, 22). L7, L15, L44, and L51 are 5,10-dideazatetrahydrofolate-resistant L1210 variants isolated by chemical mutagenesis followed by selection in the presence of this drug. In these cell lines folypolyglutamate synthetase was mutated, resulting in a marked reduction in activity of the protein, but RFC1 function was not altered (23). All cell lines were grown in RPMI 1640 medium containing 5% bovine calf serum (Hy-
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Clöne, 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO₂. For assay of thiamin accumulation, cells were grown in thiamin-free RPMI medium (custom-made by Life Technologies, Inc.) containing 5% dialyzed bovine calf serum (Life Technologies, Inc.), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) supplemented with 30 nm tritiated thiamin. For culture of R16 and T2 cells, G418 at a concentration of 750 μg/ml was included in the medium to ensure stable high level expression of RFC1.

Transport Studies—Cells were harvested, washed twice with HBS (20 mM NaCl, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose, pH 7.4), and resuspended in HBS to 1.5 × 10⁶ cells/ml. For some experiments, glucose-free HBS was also used. Cell suspensions were incubated at 37 °C for 20 min following which uptake was initiated by the addition of [³H]thiamin, [³H]TPP, or [³H]MTX, and samples were taken at the indicated times. Uptake was terminated by injection of 1 ml of the cell suspension into 10 ml of ice-cold HBS. Cells were collected by centrifugation, washed twice with ice-cold HBS, dried, and digested with 1 N NaOH. After the residual ether was removed in a speed vac, the extract was spiked with unlabeled thiamin, TMP, and TPP and separated on a reversed-phase HPLC column (Waters Spherisorb, 5 μm ODS2, 4.6 × 250 mm) as described previously (25). Separation of the thiamin, TMP, and TPP was achieved with a linear gradient of from 0 to 60% of acetonitrile in 70 mM phosphate, pH 7.4, over 30 min followed by a 10-min elution with 70 mM phosphate, pH 7.4, at a flow rate of 1 ml/min. Under these conditions, elution times of thiamin, TMP, and TPP were 20, 15.6, and 14 min, respectively. Fractions (0.5 ml) were collected in 8-ml scintillation vials, and radioactivity was assessed as indicated above. The levels of thiamin and its metabolites were normalized to units of nmol/dry weight of cells.

Accumulation of Thiamin and TPP in Cells under Growth Conditions—Cells (3 × 10⁶) grown in complete RPMI 1640 were washed twice with thiamin-free RPMI and resuspended into the same medium supplemented with 30 nm [³H]thiamin. After 1 week of exponential growth, cells were harvested, washed twice with ice-cold HBS, and processed for intracellular tritium as described for transport studies.

RESULTS

The Characteristics of Net Thiamin Uptake and Efflux in Murine Leukemia Cells That Overexpress, or Lack Functional, RFC1—Thiamin uptake was assessed in several murine leukemia cell lines with different levels of RFC1 function. MTX-A is a subline of murine leukemia L1210 cells that lack RFC1 activity due to an alanine to proline substitution at amino acid 130 (21). R16 cells, derived by transfection of RFC1 cDNA into MTX-A cells (21), express about 10 times more RFC1 than wild-type L1210 cells. As indicated in the inset of Fig. 1, MTX uptake in MTX-A cells was negligible over 30 min, whereas MTX uptake in R16 cells was very rapid and reached steady state within 10 min (22). The pattern of uptake of thiamin was reversible by cold thiamin. Net uptake in MTX-A cells was roughly three times greater than that of R16 cells by 1 h, though neither cell line was at steady state reached over the interval of observation (upper panel of Fig. 1). HPLC analysis indicated that at 1 h 90 ± 3 and 75 ± 6% (n = 2) of intracellular tritium was the active thiamin metabolite, TPP in R16 and MTX-A cells, respectively; in the latter the remainder of intracellular tritium was thiamin.

The lower panel of Fig. 1 illustrates the decline in cell tritium when MTX-A and R16 cells were loaded with 0.2 μM [³H]thiamin for 1 h prior to resuspension into thiamin-free buffer. The decrease of intracellular thiamin can be characterized by a single exponential in both cell lines, and the slope of the lines extrapolate through the time 0 points that represent the initial level of intracellular tritium. The rate constant for TPP efflux from R16 cells was 4-fold greater than from MTX-A cells, 0.58 versus 0.14 h⁻¹, respectively. However, in both cell lines the rate of loss of TPP was only a small fraction of the rate of thiamin influx (see below).

Initial Uptake of Thiamin—Initial thiamin uptake was assessed in L1210 and R16 cells over 20 s at an extracellular concentration of 0.2 μM. As indicated in the upper panel of Fig. 2, the initial uptake rate for thiamin in R16 cells was ~28% higher than in L1210 cells, much less than the 9-fold difference in RFC1 expression and MTX influx between these cell lines. Moreover, addition of 25 μM MTX, which would reduce RFC1-mediated influx by at least 70% (based on the RFC1-mediated MTX influx Ki of 7 μM (17) and a thiamin influx Ki of 2.8 mm (see below), had no effect at all on thiamin influx in either cell line. Hence, RFC1 does not contribute to thiamin influx. The very low affinity of RFC1 for thiamin was confirmed by evaluating the inhibitory effect of this vitamin on MTX influx in L1210 cells. As shown in the lower panel of Fig. 2, there was a gradual increasing inhibition of MTX influx, albeit to a small degree, as the thiamin concentration was increased from 0.1 to 1 mM. At 1 mM thiamin, MTX influx was reduced by only ~28%. Based upon the MTX influx Ki of 7 μM, the Ki for thiamin was calculated to be 2.8 mm.

TPP Influx in Murine Leukemia Cells—Since thiamin is rapidly phosphorylated to TPP in these cells, one explanation for low net thiamin uptake in cells with high level RFC1 expression (R16) is that TPP generated in the cell is a substrate for,
and is exported by, RFC1. To explore this possibility, the effect of TPP on MTX influx was assessed along with the transport properties of TPP (Fig. 3). TPP inhibited MTX influx with a Ki of 3265 mM (n = 3) in L1210 cells (upper panel), a value only 4-fold higher than the MTX influx Kt of 7 mM, consistent with a previous report (26). Furthermore, TPP influx was directly related to the level of RFC1 activity. Influx in the MTXrA cells was one-fourth that of wild-type L1210 cells, and influx was 7-fold greater in R16 cells than L1210 cells. Furthermore, 25 mM MTX markedly decreased TPP influx in R16 and L1210 cells, whereas TPP initial uptake in MTXrA cells was the same in the presence or absence of 25 mM MTX (lower panel). Hence, RFC1-mediated influx of TPP is equal to, or larger than, transport mediated by other process(es).

The Effect of Intracellular MTX on Net Thiamin Uptake—If TPP efflux is indeed mediated by RFC1, loading cells to high levels of MTX should competitively inhibit efflux of TPP and augment net TPP cellular accumulation. This was found to be the case. Incubation of R16 and MTXrA cells with 1 mM MTX resulted in intracellular MTX levels of 550 and 220 nmol/g dry wt (100 and 63 µM, respectively, based upon a ratio of intracellular water to dry weight of 3.5 µl/mg), since MTX enters cells via passive diffusion and possibly routes other than RFC1 at this high concentration. As shown in Fig. 4, intracellular accumulation of thiamin and its metabolites was doubled in R16 cells, to a level comparable to that of MTXrA cells, by pre- and co-incubation with 1 mM MTX. HPLC analysis confirmed that this increase in net uptake was due entirely to an increase in TPP accumulation. On the other hand, there was no effect of 1 mM MTX on thiamin uptake in MTXrA cells, all consistent with the lack of RFC1 function in this cell line.

Energy Dependence of Net Thiamin Uptake—When L1210, MTXrA, and R16 cells are incubated with 10 mM azide in the absence of glucose, intracellular ATP is depleted, an effect at least partially reversed by addition of 5 mM glucose to the transport buffer (5). As indicated in Fig. 5 (upper panel), net thiamin uptake in MTXrA cells was higher than that in L1210 cells without energy depletion. However, thiamin uptake in ATP-depleted cells was decreased to the same level in both L1210 and MTXrA cells. Net thiamin uptake in R16 cells was markedly lower than in L1210 and MTXrA cells regardless of the energy status. This energy dependence of thiamin accumula-
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**TABLE I  Accumulation of thiamine in murine leukemia cell lines**

| Cell line | RFC1 function* relative to L1210 cells | Thiamine accumulation | Fold change in thiamine accumulation relative to L1210 cells |
|-----------|----------------------------------------|-----------------------|----------------------------------------------------------|
| L1210     | 1.0                                    | 4.2 ± 0.3             | 1                                                        |
| R16       | 9.4                                    | 2.7 ± 0.1             | 0.64                                                     |
| T2        | 7.4                                    | 1.8 ± 0.1             | 0.42                                                     |
| L7        | 0.7                                    | 5.5 ± 0.2             | 1.3                                                      |
| L15       | 0.8                                    | 4.8 ± 0.1             | 1.1                                                      |
| L44       | 1.2                                    | 4.8 ± 0.1             | 1.1                                                      |
| L51       | 1.1                                    | 3.8 ± 0.1             | 0.90                                                     |
| G1a       | 0.026 (MTX)                            | 7.5 ± 0.3             | 1.8                                                      |
| G2        | 0.05                                   | 10.2 ± 0.5            | 2.4                                                      |
| MTXA      | 0.010                                  | 12.1 ± 0.5            | 2.8                                                      |
| D10       | 0.015                                  | 10.6 ± 0.5            | 2.5                                                      |

* Based upon MTX influx.

**FIG. 5. Effect of energy status on net thiamin uptake in MTXrA, L1210, and R16 cells (upper panel) and relative levels of intracellular thiamin and TPP in L1210 cells (lower panel).** Upper panel, MTXrA, L1210, and R16 cells were incubated in HBS or glucose free-HBS at 37 °C for 5 min before 10 mM azide was introduced into the cell suspensions. After an additional 15-min interval, 0.2 μM tritiated thiamin was added to initiate transport. Energy+ indicates cells resuspended into HBS containing 5 mM glucose, and energy− indicates cells resuspended in glucose-free HBS. Data are the means ± S.E. of three experiments. When not apparent, error bars are smaller than the symbols. Bottom panel, L1210 cells were exposed to 0.2 μM tritiated thiamin for 10 min or 1 h under the same conditions as described in the upper panel either in HBS or glucose free-HBS. Following this, intracellular thiamin and its metabolites were extracted and analyzed by HPLC. Data are the average of two separate experiments ± S.E.

Thiamin transport in murine leukemia cells is likely mediated by a facilitative carrier. Accumulation of its active coenzyme form is attributed to rapid phosphorylation of thiamin to TPP which is, to a large extent, retained within cells. This is a common phenomenon for many different substrates of the major facilitator superfamily, as occurs with phosphorylation of nucleosides (27, 28) and polyglutamation of folates (29). Consistent with this was the observation that in energy-depleted cells, phosphorylation was impaired and net thiamin uptake was markedly decreased (Fig. 5). Interestingly, the steady-state thiamin level in energy-depleted L1210 cells was ~0.3 μM (1 nmol/g dry weight), comparable to the extracellular level (0.2 μM), consistent with an equilibrating process.

Folate and thiamin, both B family vitamins, differ not only in chemical structure but also in charge. At physiological pH, folate is a bivalent anion, whereas thiamin bears one positive charge. The similarity between RFC1 and the thiamin transporter, especially in the predicted transmembrane domains, raised the possibility that these carriers may share common substrates. This was not the case. Instead, we have demonstrated that RFC1 transports the major thiamin metabolite, TPP, with an influx $K_i$ only ~4 times greater than the MTX.
influx $K_t$. This has important consequences with respect to the level of TTP accumulation. After thiamin enters cells it is phosphorylated to TTP and its molecular charge changes from positive to negative. This, in turn, is associated with an increased affinity for RFC1, an anion exchanger, that mediates TTP efflux, leading to a decrease in net intracellular TTP accumulation. Accordingly, TTP accumulation in these cells is enhanced by the rate of entry of thiamin mediated by the thiamin transporter and the rate of phosphorylation catalyzed by thiamin pyrophosphokinase. TTP accumulation is countered by the efflux of TTP mediated by RFC1. Hence, the net level is determined by balance of these processes. Since efflux studies show that the major portion of intracellular TTP is retained within the cells, there must be a large component that is bound to TTP-dependent apoenzymes in cytosol and mitochondria (30). In addition, TTP may be hydrolyzed, in part, to TMP which is, in turn, exported. TMP also appears to be a good substrate for RFC1 since TMP inhibits RFC1-mediated MTX influx with a $K_t$ of 15 µM.²

Increased thiamin accumulation associated with decreased RFC1 activity observed when cells were grown at a physiological thiamin concentration (~30 nM) suggests that the ability of RFC1 to export TTP may have important biological consequences. The requirement for thiamin is ubiquitous, but thiamin-responsive megaloblastic anemia syndrome and dietary thiamin deficiency cause tissue-specific and nonoverlapping defects (9–11). Metabolically active tissues are vulnerable to thiamin deficiency due to heavy usage of TTP-dependent enzymes. The observation that RFC1 mediates efflux of TTP may provide another important dimension to the understanding of thiamin metabolism. Tissues with high expression of RFC1 may export more TTP, making them more susceptible to metabolic derangement when thiamin is scarce. Hence, RFC1 expression may modulate the tolerance to thiamin deficiency associated with either mutations in SLC19A2, dietary deficiency, or malabsorption. It remains to be established if RFC1 is expressed in thiamin-sensitive cell types and whether metabolic defects associated with thiamin deficiency might be modified by the level of RFC1 expression.

Although TTP is not present in plasma, but accumulates in erythrocytes, TMP is present in plasma at concentrations only slightly lower than that of thiamin (31). As indicated above, TTP is likely a good substrate for RFC1, and transport by this route could be of importance under conditions in which the thiamin transporter is defective. Hence, at high blood levels of thiamin and TTP, substantial delivery of TTP via RFC1 might obviate the consequences of the loss of the thiamin transporter, another potential role for RFC1 as a modifying element in this clinical situation.

RFC1-mediated TTP transport could also play a role in thiamin absorption in intestine. Dietary vitamin B$_1$ exists predominantly as thiamin pyrophosphate that is hydrolyzed to thiamin by a phosphatase in the intestinal lumen before absorption. RFC1 is expressed in intestine and is proposed to mediate folate absorption (32, 33). It is possible that some TTP may be directly delivered into mucosal cells by RFC1 before hydrolysis to thiamin is achieved. Furthermore, TTP is derived from dephosphorylation of TPP and/or transphosphorylation of thiamin by a membrane-associated alkaline phosphatase in intestinal mucosa (34, 35). TMP has been shown to cross everted rat jejunal sac wall unchanged and enter the serosal fluid (36). It is possible that this process is RFC1-dependent.

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REFERENCES

1. Dixon, K. H., Lanpher, B. C., Chiu, J., Kelley, K., and Cowan, K. H. (1994) J. Biol. Chem. 269, 17–20
2. Williams, F. M. R., Murray, R. C., Underhill, T. M., and Flintoff, W. F. (1994) J. Biol. Chem. 269, 5810–5816
3. Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr. (1998) Microbiol. Mol. Biol. Rev. 62, 1–34
4. Sierra, E. E., and Goldman, I. D. (1999) Semin. Oncol. 26, Suppl. 6, 11–23
5. Goldman, I. D. (1971) Ann. N. Y. Acad. Sci. 186, 400–422
6. Yang, C.-H., Sirotznak, P. M., and Dembo, M. (1984) J. Membr. Biol. 79, 285–292
7. Henderson, G. B., and Zevely, E. M. (1983) Arch. Biochem. Biophys. 221, 438–446
8. Rindz, G., and Laffore, U. (1997) Methods Enzymol. 279, 118–131
9. Kril, J. J. (1996) Metab. Brain Dis. 11, 9–17
10. Mandel, H., Berant, M., Hazani, A., and Naveh, Y. (1984) N. Engl. J. Med. 311, 836–838
11. Stagg, A. R., Fleming, J. C., Baker, M. A., Sakamoto, M., Cohen, N., and Neufeld, E. J. (1999) J. Clin. Invest. 103, 723–729
12. Labas, V., Raz, T., Baron, D., Mandel, H., Williams, H., Barrett, T., Szargel, R., McDonald, L., Shalata, A., Nosaka, K., Gregory, S., and Cohen, N. (1999) Nat. Genet. 22, 300–304
13. Fleming, J. C., Tartaglini, E., Steinkamp, M. P., Schorderter, D. F., Cohen, N., and Neufeld, E. J. (1999) Nat. Genet. 22, 305–308
14. Diaz, G. A., Banikazemi, M., Oishi, K., Desnick, R. J., and Gelb, B. D. (1999) Nat. Genet. 22, 309–312
15. Dutta, B., Huang, W., Molera, M., Kekuda, R., Leibach, F. H., Devoe, L. D., Ganapathy, V., and Prasad, P. D. (1999) J. Biol. Chem. 274, 31925–31932
16. Fry, D. W., Yalowich, J. C., and Goldman, I. D. (1982) J. Biol. Chem. 257, 1890–1896
17. Zhao, R., Assaraf, Y. G., and Goldman, I. D. (1998) J. Biol. Chem. 273, 7873–7877
18. Zhao, R., Gao, F., Bahani, S., and Goldman, I. D. (2000) Clin. Cancer Res. 6, 3304–3311
19. Zhao, R., Sharina, I. G., and Goldman, I. D. (1999) Mol. Pharmacol. 56, 68–76
20. Schuetz, J. D., Matherly, L. H., Westin, E. H., and Goldman, I. D. (1988) J. Biol. Chem. 263, 9840–9847
21. Brigue, K. E., Spinella, M. J., Sierra, E. E., and Goldman, I. D. (1995) J. Biol. Chem. 270, 22974–22979
22. Zhao, R., Seither, R., Brigue, K. E., Sharina, I. G., Wang, P. J., and Goldman, I. D. (1997) J. Biol. Chem. 272, 21207–21212
23. Zhao, R., Titus, S., Gao, F., Moran, R. G., and Goldman, I. D. (2000) J. Biol. Chem. 275, 26599–26606
24. Matsuda, T., and Cooper, J. R. (1981) Anal. Biochem. 117, 203–207
25. Barile, M., Valenti, D., Brizio, C., Quagliariello, E., and Passarella, S. (1998) FEBS Lett. 435, 45–10
26. Henderson, G. B., and Zevely, E. M. (1985) J. Membr. Biol. 85, 263–268
27. Bowen, D., Diasio, R. B., and Goldman, I. D. (1979) J. Biol. Chem. 254, 5333–5339
28. Cuibelski, R. L., Fry, D. W., and Goldman, I. D. (1982) Biochim. Biophys. Acta 714, 435–441
29. Shane, B. (1989) Vitam. Horm. 45, 263–335
30. Bettendorf, L. (1995) Neurochem. Int. 26, 295–302
31. Tallaksen, C. M., Bohmer, T., Karlson, J., and Bell, H. (1997) Methods Enzymol. 279, 67–74
32. Chiao, J. H., Roy, K., Tolner, B., Yang, C. H., and Sirotznak, P. M. (1997) J. Biol. Chem. 272, 11165–11170
33. Kumar, C. K., Nguyen, T. T., Gonzalez, F. B., and Said, H. M. (1998) Am. J. Physiol. 274, C289–C294
34. Rindi, G., Ricci, V., Gastaldi, G., and Patrini, C. (1995) Arch. Physiol. Biochem. 103, 33–38
35. Matsuda, T., Maeda, S., Baba, A., and Iwata, H. (1978) J. Nutr. Sci. Vitamino 24, 123–132
36. Patrini, C., Reggiani, C., Laforenza, U., and Rindi, G. (1988) J. Neurochem. 50, 90–93

² R. Zhao, F. Gao, Y. Wang, G. A. Diaz, B. D. Gelb, and I. D. Goldman, unpublished results.
