ENDOCYTOSIS AND RECYCLING OF THE T3–T CELL RECEPTOR COMPLEX

The Role of T3 Phosphorylation

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The specific recognition of antigen by T cells is mediated by the disulfide-linked, heterodimeric T cell receptor (TCR) glycoprotein (1–3). The clonally variable α and β subunits, of ~40–50 kD, are encoded by immunoglobulin-like gene segments that rearrange during the thymic differentiation of T cells (4–8). These polypeptides are noncovalently associated on the T cell surface with the T3 molecule, which on human T cells has been defined as a group of three invariant polypeptides (γ, δ, and ε) of 20–26 kD (3, 9–12). Although the specificity of antigen recognition is controlled by the clonally variable α and β chains of the T cell receptor (13, 14), it is thought that the T3 polypeptides function in signal transduction. Antibodies to either the TCR or T3 chains may activate cells under the appropriate conditions (2, 15, 16). The initial events in this process involve phosphoinositide breakdown, a rapid rise in cytoplasmic free Ca++, and the activation of protein kinase C (17–22). Notably, one result of either antigenic stimulation or direct activation of protein kinase C using phorbol esters is the phosphorylation of components of the T3 molecule (23–25).

A striking feature of the dynamics of many cell surface receptors is their involvement in a pathway of either constitutive or ligand-induced endocytosis and recycling back to the cell surface (26–28). Such receptors cluster in coated pits, enter the cell via clathrin-coated vesicles, and pass through an acidified endosomal compartment before their reappearance at the cell surface. This pathway serves a variety of functions in different systems, including nutrient uptake, protein clearance, ligand dissociation and degradation, receptor-level regulation, protein processing, and transepithelial transport.

The factors that control the endocytosis and recycling of membrane receptors are only beginning to be understood. For some receptors the process appears constitutive (29), whereas for others it is ligand induced (30). Recent evidence has pointed to an important role for the cytoplasmic portion of membrane receptors in mediating this process. For example, endocytosis-incompetent low-density lipoprotein (LDL) receptor mutants have defects that map to this region.
of the molecule (31, 32), and studies using chimeric viral glycoprotein constructs point to this region as well (33). Nevertheless, it has been difficult to define any common features of the cytoplasmic portions of recycling receptors, the structures of which have been determined. However, a role for phosphorylation has been suggested by the observation that, for a number of receptors, phorbol ester treatment, which activates protein kinase C and induces receptor phosphorylation, induces the rapid internalization of these receptors as well (23, 30, 34, 35).

The endocytosis and recycling of membrane receptors has traditionally been studied by following the fate of bound ligand. However the TCR is a receptor for which a natural, high-affinity ligand is not generally available. To study the dynamics of this receptor, a novel approach, using cell surface iodination and treatment of intact cells with neuraminidase, was devised to assess the fate of cell surface molecules. By using this approach, it is demonstrated here that T3-TCR complexes constitutively recycle on a human T cell tumor, and that the levels of cell surface and intracellular receptors may be modulated by various means. Further, the data strongly suggest that T3-TCR recycling is dependent upon phosphorylation of the T3 γ polypeptide.

Materials and Methods

Cells. The human T leukemic cell line HPB-MLT (36) was maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 15% FCS (Hazelton Research Products, Denver, PA), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Standard Internalization Assay. This assay is loosely based on a protocol used previously by Snider and Rogers (37) to study the resialylation of recycling asialotransferrin receptors on a human erythroleukemia cell line. Typically 2–3 × 10⁷ cells were harvested by centrifugation for 7 min at 200 g, were washed twice with PBS, and were resuspended in 0.5 ml ice-cold PBS containing 1 mM magnesium chloride and 100 µg lactoperoxidase (Sigma Chemical Co., St. Louis, MO). Labelling was performed on ice by the addition of 1 mCi carrier free Na¹²¹¹ (New England Nuclear, Boston, MA) followed by six additions of 10 µl of 1:1,000-diluted hydrogen peroxide (37% solution) over the course of 0.5 h. Labelling was stopped by dilution with 10 ml ice-cold PBS saturated with tyrosine. After centrifugation at 4°C, cells were washed once with ice-cold PBS containing 1 mg/ml BSA, 1 mg/ml glucose (PBG) and 1 mM sodium iodide. They were then resuspended in the same buffer without sodium iodide, divided equally into the appropriate number of tubes (2–5 × 10⁶ cells/tube) for the particular experiment, and pelleted again. Aliquots of labelled cells were then resuspended in 2 ml RPMI 1640 supplemented with 15% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM Hepes buffer (pH 7.2), either ice-cold or prewarmed to 37°C. Cells were then either maintained on ice or incubated at 37°C for varying times, after which they were diluted by the addition of 4 ml ice-cold medium, and placed on ice. After centrifugation at 4°C, cells were washed once with ice-cold PBG and resuspended at ~5 × 10⁶ cells/ml in the same buffer. They were then either mock incubated on ice, or were neuraminidase treated on ice by the addition of 1/4-vol neuraminidase (Vibrio cholerae; 500 U/ml, Gibco) for 90 min, with occasional resuspension. Cell viability was checked at this stage by dye exclusion, and was typically 95%. Digestion was stopped by dilution with ice-cold PBGE (PBG containing 1 mM EDTA, centrifugation at 4°C, and an additional wash in the same buffer. Pelleted cells were then lysed at 0.3–1.0 × 10⁷ cells/ml in 2% Nonidet P-40 (NP-40), 10 mM iodoacetamide, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-HCl (pH 7.5), and insoluble debris was pelleted by centrifugation for 15 min at 12,000 g. Lysates were either used immediately or stored at −70°C.

Variations in this protocol included the addition of phorbol dibutyrate (PDB) (Sigma Chemical Co.) at 10 ng/ml to the cells in culture 45 min before initial harvest, and the
addition of PDB or phorbol myristate acetate (PMA) (Sigma Chemical Co.) at 10 ng/ml, calcium ionophore A23187 (Calbiochem-Behring, La Jolla, CA) at 0.5 μg/ml, and anti-T3 mAb 64.1 at 2 or 20 μg/ml, to the culture medium in which iodinated cells were incubated before neuraminidase treatment.

On occasions in which cell lysates were to be treated with neuraminidase, cells were washed once with PBG after the PBGE washes and before lysis. Cells were lysed at 5 × 10⁶ cells/ml in 1% NP-40, 10 mM iodoacetamide, 0.1 mM PMSF, 0.15 M sodium chloride, 10 mM Tris-HCl (pH 7.5), to which 1/4-vol of neuraminidase was added for 90 min on ice. The reaction was stopped by the addition of EDTA to 5 mM.

To assess reappearance at the cell surface, neuraminidase-treated cells were washed three times with PBGE to remove all traces of neuraminidase. After resuspension in culture medium either on ice or at 37°C for 15 min, subsequent washing, neuraminidase treatment, and lysis were as described above.

Antibodies and Immunoprecipitation. Monoclonal antibodies used were: T40/25 (clonotypic anti-TCR) (38), UCHT1 (anti-T3) (39), 64.1 (anti-T3) (40), 5E9 (anti-TIR) (41), 10.2 (anti-T1) (42), and 187.1 (anti-mouse κ chain) (43). Immunoprecipitation and washing conditions were as described previously (44).

Gel Electrophoresis. Analyses of polypeptides by discontinuous SDS-PAGE was as described previously (45). Nonequilibrium pH gradient electrophoresis (NEPHGE) using pH 3.5–10 ampholines was carried out essentially as described (46, 47).

Autoradiography and Densitometry. Radioactive species were detected in dried polyacrylamide gels by autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY) in conjunction with an intensifying screen. Densitometry was performed using preflooded film and a Quick Scan R + D densitometer (Helena Laboratories, Beaumont, TX). Because the TCR-α digested and protected peaks were not completely resolved, the percentage of internalized TCR-α relative to the total was calculated as follows: Each scan tracing was divided with a vertical line from the apex of the peak representing the digested material into a leading and trailing region. The percentage of material on the trailing shoulder was then calculated as 100 × (trailing − leading)/(trailing + leading), with the values normalized to zero at zero time, to compensate for any asymmetry in the peak. The mean and the range of three calculations derived from independent scans of the same autoradiogram are presented in each case.

Alkaline Phosphatase Treatment. Immunoprecipitates were eluted from the immunoadsorbent by boiling in 20 μl 1% SDS, and the eluates were diluted with nine volumes of 1% NP-40, 1 mM zinc chloride, 1 mM magnesium chloride, 0.1 M glycine-NaOH (pH 9.5). These were then incubated for 4 h at 37°C in the presence of 0.17 U bacterial alkaline phosphatase (Pharmacia Fine Chemicals, Piscataway, NJ), or mock incubated. Protein was recovered by the addition of 10 μg lysozyme, five volumes of acetone, and 1/10 volume 50% trichloroacetic acid, followed by overnight incubation at −20°C. After centrifugation for 15 min at 12,000 g, pellets were washed twice with ice-cold acetone, dried, and prepared for NEPHGE.

Phosphate Labelling. Cells were labelled using carrier-free [32P]orthophosphate (New England Nuclear) essentially as described (23).

Results

Experimental Approach. Because of the absence of a natural, high-affinity ligand for the T3-TCR complex, receptor dynamics were assessed by following the complexes directly. The assay developed is loosely based on a protocol used previously to study certain aspects of transferrin receptor dynamics (37). Briefly, it involves: (a) radiolabelling of cells at 0°C via lactoperoxidase-catalyzed iodination; (b) incubation of labelled cells either on ice or at 37°C for various times; (c) treatment of cells at 0°C with a large excess of neuraminidase; (d) extensive washing of cells followed by detergent lysis; (e) immunoprecipitation followed by either SDS-PAGE or two-dimensional gel electrophoresis.
FIGURE 1. Neuraminidase protection defines intracellular TfR and TCRα. Surface-iodinated HPB-MLT cells were either kept on ice continuously (lanes 1–3) or incubated at 37°C for 60 min and then returned to ice (lanes 4–6). Subsequent treatments were: 1 and 4, detergent lysis; 2 and 5, neuraminidase treatment followed by detergent lysis; 3 and 6, detergent lysis followed by neuraminidase treatment. In all cases, lysates were used for immunoprecipitation with a mixture of mAbs T40/25 (anti-TCR) and 5E9 (anti-TfR). Analysis was by 7.5% SDS-PAGE.

The rationale of this approach is that the fate of cell-surface glycoproteins that are initially labelled by iodination on ice can be assessed after incubation at 37°C by judging their subsequent sensitivity to neuraminidase digestion. Those molecules that remain on the cell surface will be desialylated by the enzyme, resulting in a shift in mobility that can be detected by either of the electrophoretic techniques noted. Those molecules that are protected from neuraminidase digestion are presumed to have moved from the cell surface to an intracellular compartment. The validity of these assumptions is demonstrated in the control experiments presented below.

The T leukemia cell line HPB-MLT (36) was used in these studies because of the availability of clonotypic mAb T40/25 (38), which recognizes the TCR heterodimer on these cells. The mAb 5E9 (41), which recognizes the human transferrin receptor (TfR), was also included as a control in most experiments, because endocytosis and recycling of this receptor has been characterized in detail (30, 48, 49).

Constitutive Endocytosis of the TCR on HPB-MLT Cells. An initial experiment to establish the validity of the experimental protocol outlined above is presented in Fig. 1. Iodinated cells that were held continuously on ice and then incubated either in the absence (lane 1) or presence (lane 2) of neuraminidase displayed an easily discernible difference in the SDS-PAGE mobility of the TfR polypeptide. Furthermore, no undigested material was apparent in the neuraminidase-treated sample. This finding is consistent with the expectation that at this temperature, no TfR internalization, and thus no protection, should occur. If iodinated cells were warmed to 37°C instead of held on ice, the TfR mobility was unchanged (Fig. 1, compare lanes 1 and 4). However, neuraminidase digestion of the warmed cells revealed that more than half of the TfR molecules were protected from digestion (lane 5). That this protection is likely due to internalization was
demonstrated by the finding that complete digestion could be obtained if the cells were lysed in detergent before neuraminidase digestion (lane 6). The dramatic protection of the TfR is consistent with previous studies indicating that in the presence of transferrin-containing serum, the equilibrium pool of intracellular TfR is greater than the cell surface pool (30, 48, 49).

Similar analysis of the TCR α and β chains reveals a clear, neuraminidase-dependent shift in the electrophoretic mobility of the former, but not the latter (Fig. 1, lanes 1 and 2). The extent of this shift is most likely dependent on the number of complex oligosaccharides carried by the different glycoproteins. This number is probably three for TfR (50, 51) and five for TCR α (8), but only two for TCR β (4). Thus, SDS-PAGE analysis is informative for detection of TfR and TCR α internalization, but is not informative for TCR β. As for the TfR, incubation at 37°C before neuraminidase digestion results in the generation of a population of TCR α molecules that are protected from digestion (Fig. 1, compare lane 5 with lanes 2 and 6). However, the fraction of TCR α molecules protected is much lower than for the TfR.

The detailed kinetics of internalization were investigated in experiments such as that presented in Fig. 2. In cells warmed to 37°C for varying lengths of time, it is clear that internalization is both temperature and time dependent. Levels of internalized TfR and TCR α rise rapidly immediately after warming, and plateau by ~1 h (Fig. 2, A, C, and D). In each case, the initial rise is presumed to represent the “in rate,” and the plateau level the equilibrium distribution of intracellular and extracellular species. Densitometric quantitation reveals that at equilibrium, as much as 15% of TCR α is found intracellularly (Fig. 2, D and E). However, in the same experiment, internalization of the T1 glycoprotein could not be detected, even though a clear neuraminidase-dependent mobility shift was observed (Fig. 2B). Thus, like the well-characterized TfR, TCR α is selectively endocytosed by HPB-MLT cells. Although the equilibrium pool size of intracellular TfR molecules is substantially greater than that of TCR α, it is known that TfR internalization is greatly enhanced in the presence of transferrin, which should be present in the fetal calf serum used in these experiments (30). In repeated experiments, lysosomotropic agents such as monensin and ammonium chloride had no effect on the internalization of either the TfR or TCR α (data not shown).

Although results qualitatively similar to those displayed in Fig. 2 have been obtained on numerous occasions, quantitative variation has been apparent. On some occasions, receptor internalization lagged after warming, and plateau values were lower than those described above. In all such cases, diminished internalization of TCR α was paralleled by diminished internalization of TfR (see, for example, Fig. 5). Hence, although cell viability was typically >95%, it is assumed that some variability in the state of the cells affected their ability to rapidly internalize cell surface molecules. Because of the extensive manipulation of cells in the assay used in this study, the present experiments are probably more likely to underestimate rather than overestimate the rate and extent of internalization of TCR α.

Recycling of Internalized TfR and TCR α to the Cell Surface. The kinetics of internalization presented in Fig. 2 suggest that within 1 h, iodinated molecules
FIGURE 2. Kinetics of TfR, T1, and TCRα internalization. Surface-iodinated cells were either held on ice or warmed for varying lengths of time and then returned to ice, and were treated with neuraminidase as indicated. After detergent lysis, immunoprecipitation was with: A, mAb 5E9 (anti-TfR); B, mAb 10.2 (anti-T1); C, mAb T40/25 (anti-TCR). Analysis was by 7.5% SDS-PAGE (A and B) or 7–15% gradient SDS-PAGE (C). D shows the results of densitometric quantitation of TCRα internalization in the experiment presented in C. E shows sample densitometric scans used in this quantitation. T denotes the trailing portion of the TCRα peak, and L denotes the leading portion. Protection was determined as described in Materials and Methods.

equilibrate between intracellular and extracellular pools. Direct evidence for a dynamic equilibrium involving the recycling of internalized TfR and TCR α molecules to the cell surface could be obtained as follows (Fig. 3). Iodinated cells were incubated for 90 min at 37°C to reach equilibrium, and were then treated
FIGURE 3. Internalized Tfr and TCRα recycle to the cell surface. Surface-iodinated cells were either kept on ice (lanes 1 and 2) or warmed to 37°C for 90 min (Warm 1) in the absence (lanes 3-6) or presence (lanes 7-10) of 2 μg/ml purified 64.1 (anti-T3) mAb. Neuraminidase treatment on ice (Neur 1), return to culture for 15 min at 37°C (Warm 2), and retreatment with neuraminidase on ice (Neur 2) were as indicated. After detergent lysis, immunoprecipitation was with: A, mAb 5E9 (anti-Tfr); B, mAb T40/25 (anti-TCR). Analysis was by 7.5% SDS-PAGE (A) or 7-15% gradient SDS-PAGE. Densitometric quantitation reveals that there is no change in the level of intracellular TCRα in the absence of the second incubation at 37°C (Warm 2). However, ~30% of the basally internalized TCRα and 60% of the 64.1-induced internalized TCRα return to the cell surface during this incubation.

with neuraminidase to allow SDS-PAGE distinction between intracellular (protected) and cell surface (digested) molecules (Fig. 3, A and B, lanes 3). An identical, further treatment with neuraminidase did not change the protection pattern (Fig. 3, A and B, lanes 4). If, after the initial neuraminidase incubation, cells were warmed for 15 min, no change in the protection pattern could be observed (Fig. 3, A and B, lanes 5). However, when the rewarmed cells were retreatred with neuraminidase, levels of protected Tfr and TCRα were seen to decrease (Fig. 3, A and B, lanes 6). This is taken as evidence that the previously protected, internal populations recycled back to the cell surface. Densitometric quantitation reveals that 30% of the internal TCRα returned to the cell surface during this time. The detailed kinetics of movement to the cell surface have not been assessed further.

Perturbation by Ligand Binding and Phorbol Ester Treatment. Under appropriate conditions, antibodies to T3 can activate T cells (15) and can, in addition, lead to modulation of the T3-TCR complex and subsequent nonresponsiveness (52). It is thought that the interaction of such ligands with the complex may mimic physiological interactions with antigen. Thus we wished to determine whether antibodies to T3 might alter the basal pattern of TCRα recycling on HPB-MLT cells.

When iodinated cells were incubated at 37°C in the presence of saturating or near-saturating amounts of anti-T3 mAb 64.1 (40), a dramatic increase in
amounts of internalized TCR α was observed, whereas internalization of TfR and T1 was unaffected (Fig. 4, A–C and data not shown). This was reflected in increases in both the initial rate of TCR α internalization and the size of the intracellular pool. There was no evidence for degradation of internalized molecules over the 3-h period examined. Furthermore, the internalized TCR α in 64.1-treated cells still recycled to the cell surface, possibly with antibody bound. Thus, in an experiment similar to that described above, at least 60% of the molecules internalized under these conditions returned to the cell surface during a 15-min period (Fig. 3, lanes 7–10). Hence, the effect of ligand may be viewed as a perturbation of the kinetic and equilibrium parameters describing the constitutive endocytosis and recycling of T3-TCR complexes on these cells.

Stimulation of T cells can result in both a rise in cytoplasmic free Ca²⁺, and the activation of protein kinase C (17–21). The former can be effected by the Ca²⁺ ionophore A23187, and the latter by phorbol esters. In conjunction, these two agents are sufficient to induce activation (17, 19, 22). To examine the effects of these agents on T3-TCR dynamics, iodinated cells were incubated at 37°C in the presence of phorbol ester and calcium ionophore, either alone or in combination. Phorbol dibutyrate at 10 ng/ml induced the internalization of more than half of the TCR α (Fig. 5B) and virtually all of the TfR (Fig. 5A). Notably, this occurred despite the fact that in this particular experiment, the untreated cells displayed particularly low levels of constitutive internalization of both molecules (compare Fig. 5 with Figs. 2 and 3). PMA treatment produced a similar effect (data not shown). However, incubation in the presence of 0.5 μg/ml A23187 had no effect on TCR α or TfR internalization, and did not potentiate the effect of phorbol esters when administered in conjunction with them (data not shown).

**Endocytosis of the TCR β, T3 γ, and T3 δ Polypeptides.** The data presented to this point provide strong evidence for the endocytosis and recycling of TCR α. Because this polypeptide exists on the cell surface in a complex with TCR δ, T3 γ, T3 δ, and T3 ε, it seems likely that the entire complex cointernalized. However, the magnitudes of the neuraminidase-dependent SDS-PAGE mobility shifts for these polypeptides were not great enough to clearly assess their cointernalization with TCR α. To obtain such evidence, selected immunoprecipitates were analyzed by two-dimensional gel electrophoresis, using NEPHGE in the first dimension and SDS-PAGE in the second dimension (Fig. 6). All five components of the T3-TCR complex could be identified (Fig. 6A) by the criteria of SDS-PAGE mobility, isoelectric point, and the presence of asparagine-linked oligosaccharides (data not shown). Heterogeneity typical of variable sialic acid content was evident for TCR β, T3 γ, and T3 δ (note that TCR α did not focus well in this system). The T3 ε polypeptide, which is not glycosylated (10), focussed as a single species. No changes in electrophoretic mobility were observed when iodinated cells were cultured for 90 min at 37°C before lysis (Fig. 6, compare A and C).

When iodinated cells were held continuously on ice and treated with neuraminidase, TCR β, T3 γ, and T3 δ were all converted to more basic forms (Fig. 6B; note that T3 ε, which is unaffected by neuraminidase, serves as a useful internal marker). However the heterogeneity of these polypeptides was not completely eliminated by neuraminidase. Notably, two abundant forms of T3 γ (designated a for acidic and b for basic), two abundant forms of T3 δ, and (on
Figure 4. Modulation of internalization by anti-T3 mAb. Surface-iodinated cells were either kept on ice or were warmed to 37°C for varying lengths of time in either the presence or absence of 2 μg/ml purified 64.1 (anti-T3) mAb. After return to ice, cells were treated with neuraminidase as indicated, and detergent lysates were prepared. Immunoprecipitation was with: A, mAb 5E9 (anti-TIR); B, mAb T40/25 (anti-TCR). Analysis was by 7.5% SDS-PAGE (A) or 7–15% gradient SDS-PAGE (B). C shows the results of densitometric quantitation of TCRα internalization from the experiment presented in B, using preflashed film, as described in Materials and Methods.
lighter exposures) two abundant forms of TCR β, remained. Strikingly, when cells were cultured for 90 min at 37°C before neuraminidase digestion, a fraction of each of these polypeptides remained as the more acidic, sialylated forms (Fig. 6, compare C and D). This temperature-dependent protection from neuraminidase digestion is taken as evidence that these polypeptides were endocytosed along with TCR α. Although no information could be obtained using this approach for the nonglycosylated T3 ε chain, these data suggest that in all likelihood, the entire T3-TCR complex recycles on HPB-MLT cells.

**T3 γ Phosphorylation.** It was reproducibly observed that of the two forms of T3 γ in neuraminidase-treated cells (Fig. 6B), the more acidic, T3 γ a species was selectively protected from neuraminidase digestion (and hence selectively internalized) in cells cultured at 37°C (Fig. 6D). This suggested that the structural feature that distinguishes T3 γ a and b correlates closely with, and might be causally linked to, the endocytosis of this molecule. The following experiment was performed to define this structural feature.

Iodinated cells were held on ice and subjected to neuraminidase digestion, and, as before, displayed both the a and b forms of T3 γ (Fig. 7A). When the detergent lysate from these iodinated and neuraminidase-treated cells was redigested with an equivalent amount of neuraminidase, no change in the ratio of the two species was observed (Fig. 7B). Thus, it is unlikely that residual sialic acids distinguish the two forms. If cells were cultured in the presence of phorbol dibutyrate before iodination and neuraminidase treatment a dramatic increase in T3 γ a occurred at the expense of T3 γ b (Fig. 7C). Because phorbol ester treatment has been documented to result in phosphorylation of T3 γ (23), a possible interpretation of this finding is that T3 γ a is a phosphorylated form of T3 γ b. To address this point directly, immunoprecipitates from iodinated and
neuraminidase-treated cells were eluted from the immunoabsorbent, and either incubated with alkaline phosphatase, or mock incubated. Whereas the mock incubation had little effect, if any, on the ratio of the two forms (Fig. 7D), alkaline phosphatase completely converted the α form of T3γ to the β form (Fig. 7E). In addition, a phosphorylated form of T3γ isolated from 32P-labelled, neuraminidase-treated cells was found to comigrate with T3γα (Fig. 7F), a conclusion supported by mixing experiments (data not shown). Thus, both phosphorylated and nonphosphorylated forms of the T3γ polypeptide exist in HPB-MLT cells. Whereas the phosphorylated molecules are constitutively internalized, the nonphosphorylated molecules appear to be expressed stably on the cell surface.

Discussion

This report describes an approach to assess the dynamics of cell surface glycoproteins, in which neuraminidase digestion of intact cells is used to determine the fate of cell surface molecules initially labelled via lactoperoxidase-catalyzed iodination on ice. This approach is particularly advantageous because
FIGURE 7  Phosphorylation of T3 γ. A–E: Cells cultured in the absence or presence of 10 ng/ml PDB for 45 min at 37°C were surface iodinated and subsequently neuraminidase treated on ice. Detergent lysates were immunoprecipitated directly with a mixture of mAbs T40/25 (anti-TCR) and UCHT1 (anti-T3), or were treated with neuraminidase before immunoprecipitation. Eluates from the immunoadsorbent were analyzed directly by electrophoresis, or were alkaline phosphatase treated or mock incubated before analysis. Alkaline phosphatase–treated and mock-incubated samples were recovered by acetone/TCA precipitation. Analysis was by two-dimensional gel electrophoresis, NEPHGE in the first dimension and 10% SDS-PAGE in the second dimension. A, neuraminidase-treated cells; B, neuraminidase-treated cells, lysate treated with neuraminidase; C, PDB-induced, neuraminidase-treated cells; D, neuraminidase-treated cells; E, neuraminidase-treated cells; immunoprecipitate mock incubated; F, neuraminidase-treated cells; immunoprecipitate incubated with alkaline phosphatase. F, cells preloaded with [32P] orthophosphate were cultured for 15 min in the presence of 10 ng/ml PDB. After washing and neuraminidase treatment, detergent lysis, immunoprecipitation, and two-dimensional gel electrophoresis were carried out as above.
it simultaneously allows direct visualization of both cell surface and internalized molecules, and does not rely upon ligand binding. Thus internalization can be followed in the absence of any ligand, and the effects of ligand binding can be carefully assessed. Furthermore, this approach should in theory be useful to assess the dynamics of other sialylated glycoproteins for which specific antibodies are available for analysis.

Using this technique, it has been possible to document the constitutive endocytosis and recycling of the T3-TCR complex on a human T cell tumor. Furthermore, evidence has been obtained that T3-TCR internalization is tightly coupled to, and may be controlled by, T3 phosphorylation. Stable populations of both phosphorylated and nonphosphorylated T3 γ peptides have been identified in HPB-MLT cells. Whereas the former are constitutively endocytosed, the latter appear to be excluded from this pathway, and remain stably on the cell surface. Hence it seems likely that only those T3-TCR complexes that contain phosphorylated T3 γ peptides constitutively recycle on these cells. Activators of protein kinase C induce additional T3 γ phosphorylation and increase internalization of T3-TCR complexes. On the other hand, a ligand to the T3-TCR complex (mAb 64.1) induces additional internalization without affecting the level of T3 γ phosphorylation (data not shown). Thus phosphorylation may be a specific signal for receptor internalization, and not the converse. However the present data cannot rule out the possibility that nonphosphorylated molecules are endocytosed at very low levels, rather than being absolutely excluded from this pathway.

In a number of systems, it has been possible to establish a correlation between receptor phosphorylation and receptor internalization, based on the finding that both phenomena are stimulated by either a specific ligand or by a phorbol ester (23, 30, 34, 35). However, a direct demonstration that the phosphorylated polypeptides are selectively internalized has not previously been presented. Due to the relationship between phosphorylation and internalization in other systems, it seems likely that receptor phosphorylation may be a general signal for internalization. However, although phosphorylation may be necessary for internalization and may provide one level of control, it may not by itself be a sufficient signal. Thus, additional structural features of the polypeptide may be important in this process, and additional levels of control may exist. Notably, the superinduction of class I antigen phosphorylation by treatment with phorbol esters does not result in a detectable loss of these molecules from the cell surface (23).

By labelling cells with [32P]orthophosphate, phosphoserine has been identified in murine T3 peptides after either antigenic stimulation or phorbol ester induction (25), and has been identified in the human T3 γ peptide after phorbol ester induction (53). Further, a potential site for protein kinase C-mediated phosphorylation has been identified in the presumed intracytoplasmic region of the human T3 γ peptide (54). It is likely that the site of T3 γ phosphorylation is identical in both uninduced and phorbol ester-induced HPB-MLT cells, because two-dimensional gel electrophoresis reveals no doubly phosphorylated molecules in induced cells (Fig. 7, A and C). However it is striking that, although typically 30–50% of the T3 γ is basally phosphorylated in HPB-MLT, as judged by two-dimensional gel electrophoresis of iodinated material, the peptide cannot be
labeled with $[^{32}\text{P}]$orthophosphate in the absence of phorbol ester induction (23; and data not shown). This suggests that phosphate turnover is minimal, and that recycling is not coupled to phosphate turnover per se. Whether and how phosphate turnover is controlled remains obscure, but it should be noted that if iodination and neuraminidase treatment of HPB-MLT is performed at room temperature instead of on ice, the phosphorylated T3 $\gamma$ peptides cannot be detected (data not shown).

It will be particularly important to investigate the role of ligand valency in modulating T3-TCR recycling and to understand the relationship of this phenomenon to antibody induced T3-TCR modulation. Under the conditions used in this study, mAb 64.1 induces additional internalization (Fig. 4) but does not induce additional T3 $\gamma$ phosphorylation (data not shown). It is possible that anti-T3 mAbs specifically increase internalization of only phosphorylated T3-TCR complexes, or alternatively, induce the internalization of nonphosphorylated complexes as well. Ligand-induced crosslinking of nonphosphorylated complexes to each other may allow them to be efficiently endocytosed. Alternatively, crosslinking of nonphosphorylated to phosphorylated T3-TCR complexes may allow the former to "piggyback" through the recycling pathway. Regardless, T3-TCR complexes internalized in the presence of mAb 64.1 clearly recycle back to the cell surface (Fig. 3), and in repeated experiments, no convincing evidence for receptor degradation has been obtained during the 3-h period examined. In contrast, it has been demonstrated that Fc receptors that bind monovalent ligand are endocytosed and recycle back to the cell surface, whereas multivalent ligand-receptor complexes are shunted to lysosomes, where they are degraded (55, 56).

It may be the case that over periods of time longer than those analyzed here, T3-TCR degradation may occur, and this may be the basis for antibody-induced T3-TCR modulation. This view of T3-TCR modulation would be consistent with that of Rinnooy Kan et al., (57) but opposed to that of Reinherz et al., (52), who reported external shedding of T3 under the conditions of modulation.

What is the functional significance of T3-TCR recycling? Although 30–50% of the T3 molecules are phosphorylated in the tumor cell line HPB-MLT, this number may be much lower in normal, resting cells. Because the stimulation of T3 phosphorylation appears to be a consequence of T cell activation, T3-TCR recycling may therefore be strictly a property of activated cells. Because both receptor modulation and antigen unresponsiveness can be induced by specific antibody (52), by supraoptimal doses of antigen (58, 59), and by phorbol esters (60), physiologically important regulation of T cell function may be mediated through control of receptor levels. It is therefore possible that T3-TCR recycling affords the cell a means to modify cell surface receptor density, much in the same manner that other receptors may be downregulated in response to ligand binding.

Recycling may, however, play a more important role in ligand uptake and/or processing. It may provide a mechanism whereby bound material can be internalized and degraded, allowing free receptors to reappear on the cell surface. In addition, it may allow for the expression of processed antigen on the T cell surface, which might then function in mediating antigen-specific T-T interactions. This suggests analogy with B cell surface immunoglobulin, which is thought
to mediate the uptake of specific antigen for processing and presentation to helper T cells, allowing for antigen-specific T-B interactions (61, 62). Although these proposals are purely speculative, it seems certain that a more complete understanding of T3-TCR recycling will help to shed light on novel aspects of the control of T cell function.

Summary

An assay has been developed to assess the dynamics of cell surface glycoproteins, in which neuraminidase digestion of intact cells is used to determine the fate of cell surface molecules initially labelled via lactoperoxidase-catalyzed iodination. This approach has been used to demonstrate the constitutive endocytosis and recycling of the T3-T cell receptor (TCR) complex on the human T leukemic cell line HPB-MLT. Stable populations of both phosphorylated and nonphosphorylated forms of the T3 γ peptide have been identified in these cells. Whereas the former are constitutively endocytosed, the latter appear to be excluded from this pathway. The results presented indicate that T3 γ phosphorylation may control the endocytosis and recycling of the T3-TCR complex on this cell line.

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