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Luminescence Quenching by Long Range Electron Transfer: A Probe of Protein Clustering and Conformation at the Cell Surface

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Quenching of luminescence from fluorescent and phosphorescent probes by nitroxide spin labels with a long range electron transfer (LRET) mechanism (44,45) has been tested as a tool to monitor association/clustering and conformational changes of cell surface proteins. The membrane proteins were labeled with monoclonal antibodies or Fab fragments conjugated with luminescent probes or water-soluble nitroxide spin labels. The method was tested as a probe of 3 different aspects of protein-protein association involving class I MHC molecules: (1) interaction between the heavy and light chains of the MHC molecules, (2) clustering, self-association of MHC molecules, (3) proximity of MHC molecules to transferrin receptors of fibroblasts or surface immunoglobulin molecules of B lymphoblasts. The extent of quenching upon increasing the fractional density of the quencher was sensitive for protein association in accordance with earlier immunoprecipitation and flow cytometric Förster-type energy transfer (FCET) data obtained on the same cells. These data suggest that the LRET quenching can be used as intra- or intermolecular ruler in a 0.5–2.5 nm distance range. This approach is simpler (measurements only on donor side) and faster than many other experimental techniques in screening physical association or conformational changes of membrane proteins by means of spectrofluorimetry, flow cytometry, or microscope based imaging. © 1995 Wiley-Liss, Inc.

Key terms: Fluorescence quenching, protein association, clusters, electron transfer, flow cytometry

Association of membrane proteins may result in formation of local assemblies, clusters of proteins on the cell surface, or in organelle membranes (9,10,13,15,16, 26–28). These assemblies may be relatively short-lived, induced by external triggering molecules (14,15,55), or long-lived, mediated by cytoskeleton (4) or by membrane cholesterol (52). Formation and persistence of some assemblies may be genetically determined (38,54). There are several examples of functional significance of protein clustering, particularly in immunology (5–7,10,13–15,42,50,51,58,59,65).

Given the functional significance of protein clusters it is important to probe their formation and dissolution in living cells. Due to the resolution limits of conventional light microscopy, the majority of current techniques for detecting protein clusters on living cells are spectroscopic and based on variations of Förster-type (dipole-dipole) resonance energy transfer (21,31–33,43,46,56, 57,60,63). The efficiency of Förster-type energy transfer, a parameter dependent upon proximity of donor- and acceptor-bearing molecules, is determined from either steady-state or time-resolved intensity measurements or from a comparison of the bleaching kinetics of donor fluorophores in the absence and presence of acceptors (31).

Earlier it was shown that some molecules with electron spin multiplicities > 0, such as nitroxide radicals, are effective quenchers of excited electronic states of a wide variety of luminescent probes (2,30,34,43,45). A family of lipid soluble nitroxide quenchers already has a wide range of applications in studying protein and lipid organization in membranes (3,37). Matko et al. (43–45) showed that quenching of various luminescent probes by water soluble nitroxide quenchers is dominated by a long range electron transfer (LRET) mechanism. This finding, in accordance with other recent LRET observations (1,29,36,41,45,64), suggests that LRET between electron donors and acceptors carried by Fab fragments of monoclonal antibodies may be a potentially useful and rela-

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tively simple tool for detecting association of the labeled cell surface proteins or, in case of intramolecular labeling, to study conformational changes in the extracellular domains of large membrane proteins. Application of small water-soluble nitroxide quenchers has the advantage of minimal conformational perturbation of antibodies and avoidance of non-specific binding often appearing in the case of relatively hydrophobic fluorescent acceptors (*e.g., rhodamine*).

Here we report a detailed analysis on the application of this LRET quenching (LRETQ) at the surface of several cell lines using both spectrofluorimetry and flow cytometry, a comparison with data obtained by other techniques as well as a discussion of the advantages and drawbacks of this approach.

MATERIALS AND METHODS

Chemicals

Fluorescein isothiocyanate (FITC), sulfo-rhodamine 101 (Texas Red), and fluorescein-conjugated transferrin were purchased from Molecular Probes (Eugene, OR). $TbCl_3$ hexahydrate (99.9%) is a product of Aldrich Chemical Co. (Milwaukee, WI). 4-hydroxy-TEMPO and 3-[2-(2-isothiocyanatoethoxy)-ethyl-carbamoyl] PROXYL (ITC-EECP) were obtained from Sigma Chemical Co. (St. Louis, MO). The Tb^{3+}-chelating agent (S)-4-[2,3-[Bis(carboxymethyl)aminopropylphenylisothiocyanate (CITC) was a kind gift of Dr. Claude Meares (University of California, Davis).

Monoclonal Antibodies

Monoclonal antibodies reactive with HLA-A,B,C (W6/32 and KE-2), HLA-A2 (BB7.2), β-2 microglobulin (BBM.1), and SIg (TB28–2) were prepared from culture supernatants of hybridomas. IgG molecules were purified by the procedure described by Ey et al. (19). Fab fragments were obtained by papain digestion and purified by Sephadex G-100 chromatography as described earlier (18).

Antibody Labeling

Purified IgG or Fab fragments were labeled with isothiocyanate derivatives of fluorescein (FITC), the Tb^{3+}-chelator (CITC) and the nitroxide spin label (EECP-ITC). Antibodies (2–4 mg/ml) were mixed with a twenty-fold molar excess of the appropriate isothiocyanate in 0.1 M sodium bicarbonate buffer (pH: 9.0) and incubated overnight at 4°C. Unconjugated label was removed by gel-filtration through a Sephadex G-50 column (1.2 × 30 cm) equilibrated with 10 mM Hepes buffer (pH: 7.4, 145 mM NaCl, 5 mM KCl). The conjugation of antibody with EECP-ITC was monitored by recording ESR absorption spectra of the conjugates. Conjugates of antibodies with fluorescein and Texas-Red were monitored by measuring the absorbance of the conjugate at 280 and 494 or 596 nm, respectively. A molar extinction coefficient of $6.3 \times 10^4\ M^{-1}\ cm^{-1}$ at 494 nm (11) was used for FITC conjugates while $8.5 \times 10^4\ m^{-1}\ cm^{-1}$ (Handbook of Molecular Probes) was used for Texas-Red. Complexing of antibodies with Tb^{3+} after CITC labeling was carried out as described earlier (49). Conjugates with a dye to protein labeling ratio between 1 and 3 were used throughout the measurements. The affinity and specificity of the labeled antibodies were tested by competition assay with unlabeled antibodies. The labeled antibodies were centrifuged for 30 min, at 100,000 rpm before labeling cells.

Cell Culture

The human B lymphoma cell line, JY [HLA-A2, B7, DR4, DQw1.3], was originally described by Terhorst and coworkers (62). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS. The cell cultures were split at 2-day intervals in order to maximize log phase growth.

Transformed human fibroblast cells, VA-2 (42), were cultured in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 5% FCS. The cells were split every 4 days to ensure a constant growth phase. Cells were removed from culture dishes with a gentle agitation by chicken serum/trypsin/collagenase (22) and then washed with fresh minimal essential medium (MEM).

Labeling of Cells With Fluorophore- and Nitroxide-Conjugated Antibodies for LRETQ Measurements

Cells were labeled with a mixture of fluorophore-KE2, quencher-KE2, and unlabeled KE2 antibodies by incubation for 45 min on ice. The labeling was usually done at a cell density of about $3–4 \times 10^6/cm^3$. The fractional amount of fluorophore-labeled antibody was kept constant (75 pmol Fab/10^6 cells) while the fractional amount (cell surface density) of quencher was varied by changing the ratio of unlabeled and quencher-labeled antibodies in the incubation mixture while keeping total antibody concentration at 150 pmol Fab/10^6 cells in cell suspensions of fixed, small volume (~ 50 μl). The quencher-antibody/fluorophore-antibody molar ratio changed between 0 and 1. The total amount of antibody was chosen so as to saturate binding sites. The equal competition between the different labeled antibodies was checked by measuring the fluorescein-emission of the cells preincubated with the same amount of unlabeled and EECP- or CITC-labeled KE2 antibodies. The unquenched level of fluorescence, as an internal control, was measured in the presence of quencher-conjugated antibodies and 10 mM ascorbate which completely inactivates the nitroxide quenchers (45). This intensity was used as F_0 in the quenching experiments, reducing the potential error from an under- or over-estimation of protein (IgG or Fab) concentration. Both the unquenched intensity (F_0) and, the quenched intensities ($F_1 \ldots F_n$) were derived as means of fluorescence histograms after subtraction of the mean belonging to the unlabeled cell (autofluorescence) background.

After incubation, the cells were washed twice with Hepes/Hank's medium (pH 7.4), followed by a centrifu-

gation for 5 min at 1,000 rpm (at 4°C) before recording flow cytometric histograms or steady-state emission spectra. Occasionally the labeled cells were fixed before measurements by incubation with 2% formaldehyde, in PBS (pH 7.3), for 20 min on ice. After labeling, the cells were stored on ice and the measurements carried out at low temperature to minimize internalization of the labeled antigen-antibody complexes. The label was confined to the cell surface; treatment of labeled cells with a pH 4.5 buffer reduced the fluorescence to the level of autofluorescence of unlabeled cells.

Spectroscopic Measurements

Fluorescence spectra and steady-state intensities were recorded in an SLM 8000 fluorimeter equipped with a temperature controlled stirred cell holder and interfaced to an IBM AT microcomputer. The fluorescence intensities were corrected using a rhodamine standard quantum counter. Fluorescein conjugates were excited at 490 nm and the emission spectrum centered at 530 nm was collected from 500 to 560 nm. For Tb^{3+}-conjugates, an excitation wavelength of 295 nm was used and the emission was monitored between 520 and 580 nm. The emission spectrum of the Tb^{3+} labeled antibodies and cells showed a maximum at 545 nm. The fluorescence intensities of the samples were derived by integrating the luminescence peaks at 520 nm and 545 nm, respectively, after background subtraction. The temperature of the samples was controlled within ± 0.1°C. The fluorescence quenching data were analyzed with a program based on the Stern-Volmer theory (35) after the necessary corrections described earlier (47). The ESR spectra of nitroxide molecules, labeled antibodies, and cells were recorded on an ESR spectrometer in the laboratory of B. Gaffney (Dept. of Chemistry, The Johns Hopkins University, Baltimore) at ~ 9 GHz microwave frequency using 100 kHz modulation frequency, at room temperature.

Flow Cytometric Measurements

The intensity of fluorescence associated with labeled cells was determined in an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) connected to a CICERO microcomputer system or in a Becton-Dickinson FACStar flow cytometer excited by a Spectraphysics Stabilite 2000 Ar ion laser. Cell debris or aggregates were eliminated on the basis of dot plots of forward and 90° light scattering, while the cells were counted based on forward angle light scatter (FALS). The fluorescein fluorescence was excited using the 488 line of the argon ion lasers (at 300–400 mW). The emission was collected with a 525 nm bandpass filter. The Texas Red fluorescence was excited by a dye laser (rhodamine 6G) at approximately 100–150 mW. The Texas Red emission was collected with a 635 nm band pass filter. At least 20,000 cells were counted for each data set and the mean fluorescence intensity was determined from the central 90% of the cell population. The mean fluorescence was quantitated using calibration by a series of fluorescent standard beads from 1.3×10^4 to 4.3×10^5 molecules/bead (Flow Cytometry Standards Corp., Research Triangle Park, NC).

Förster-Type Resonance Energy Transfer (FCET) Measurements

Förster-type resonance energy transfer between fluorescein (donor) and Texas Red (acceptor) conjugated to Fab fragments of monoclonal antibodies against cell surface protein molecules was studied in cell suspensions by means of flow cytometry as described earlier (46,60,63). A dual laser (488 and 595 nm) excitation configuration on the EPICS-752 flow cytometer was used to determine energy transfer efficiency of the labeled cell populations. Before energy transfer measurements, staining level and specificity were checked by microscopic observation of ring-like membrane staining. Staining saturation as well as competition with unlabeled antibody were also tested in all cases. The cells were labeled at a high cell density ($2–3 \times 10^7/cm^3$) in a small volume with donor and acceptor labeled antibodies or with the mixture of the two (at 1:1 molar ratio) as described earlier (46,60). Four intensity-histograms were recorded with each labeled cell sample: the fluorescence emission at 525 nm and 630 nm upon 488 nm excitation, 630 nm emission, at 595 nm excitation, and forward angle light scattering. Correction factors were determined from fluorescence intensity histograms obtained with single-labeled cells as described by Tron et al. (63). Data evaluation was done according to a procedure described earlier (46) using a critical Förster distance (R_0) of 3.9 nm for FITC-TexasRed pair in calculations of proximity estimates. The labeled samples were stored in ice and measured immediately after labeling or fixed with 2% formaldehyde to avoid temperature effects. Fixation after labeling did not significantly affect the efficiency of energy transfer.

RESULTS

Labeling of Cell Surface Protein Molecules by Tb^{3+}, FITC-, and Nitroxide-Conjugated Fab Fragments of Monoclonal Antibodies

Characteristic features of labeling were examined for cell surface class I MHC molecules expressed on two cultured cell lines, VA-2 transformed human fibroblasts and JY human B lymphoblasts. Binding of KE-2 (anti-HLA A,B,C) antibody to both cell lines was analyzed in detail. The mean channel numbers of flow cytometric intensity histograms obtained with FITC-antibody labeled cells were converted into number of sites/cell using fluorescent standard beads after taking the dye:protein ratio of our antibodies into consideration. Scatchard analysis of the binding data obtained with FITC-KE-2 resulted in estimated numbers of class I MHC copies of about 2×10^6/JY cell and 9×10^5/VA-2 cell (data not shown). The principle of equal competition was also tested for FITC-, Tb^{3+}/CITC-, EECP-conjugated and unlabeled KE-2 antibody molecules on VA-2 cells. As Figure 1A shows, all

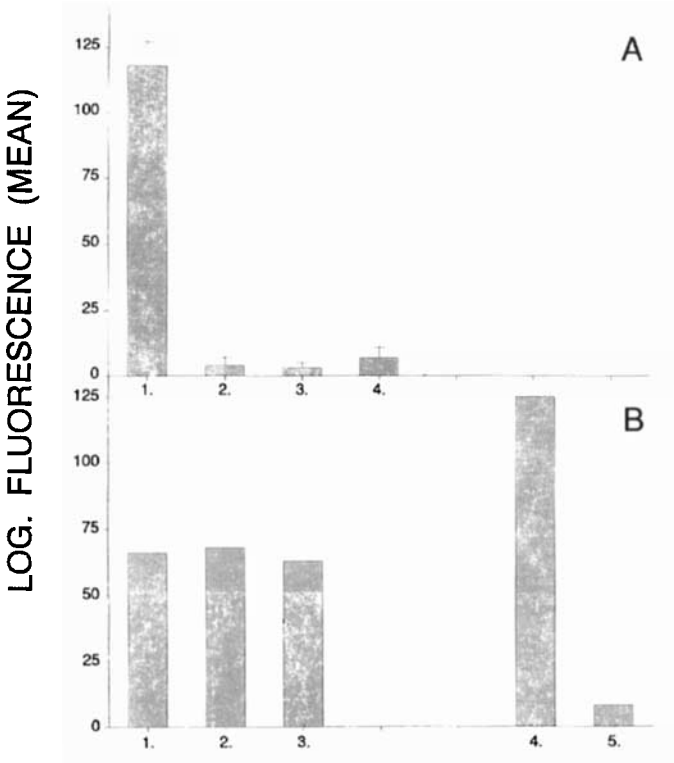

FIG. 1. Flow cytometric competition assay with different labeled and unlabeled anti-class I MHC monoclonal antibodies. **A:** Competition between labeled and unlabeled KE-2 antibodies for class I MHC molecules on the surface of VA-2 fibroblasts. The cells were incubated with saturating amount of FITC-KE2 monoclonal antibody in the absence and presence of other KE2 antibodies. The bars represent the mean of fluorescence intensity histograms recorded by flow cytometry using a logarithmic amplifier. From left to right are the values for cells labeled with FITC-KE2 alone (1), with unlabeled KE2 + FITC-KE2 (2), with CITC-KE2 + FITC-KE2 (3), and with EECP-KE2 + FITC-KE2 (4), respectively. The error bars represent the S.D. of 3–5 independent measurements. **B:** Competition between different anti-HLA antibodies for MHC class I molecules on JY lymphoblastoid cells. The cells were stained with FITC-conjugated anti-HLA A2 (BB7.2) antibodies alone (1), with unlabeled KE2 + FITC-BB7.2 (2), with unlabeled W6/32 + FITC BB7.2 (3) (left side), or with FITC-W6/32 antibodies alone (4), or with unlabeled KE2 + FITC-W6/32 (5) (right side).

three antibodies competed practically completely and equally with FITC-conjugated antibody for class I MHC molecules. Both cell lines showed a significant autofluorescence upon excitation by the 488 nm laser beam. The autofluorescence, however, did not change with increasing fractional density of quencher-conjugated antibody on the cell surface (see Fig. 5).

Relationships between different epitope-specific antibody-binding sites were examined on JY cells which have a high cell surface density of class I MHC molecules. Anti-HLA-A2 antibodies competed neither with KE-2 nor with W6/32 antibodies developed against monomorphic determinants (HLA A,B,C) of the HLA antigen, while KE-2 and W6/32 competed perfectly with each other (Fig. 1B).

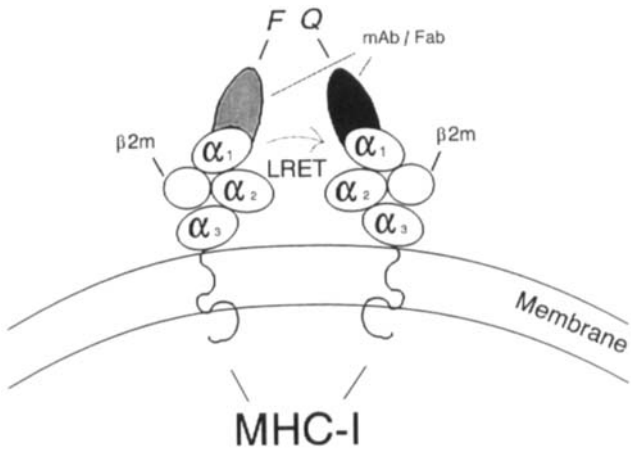

FIG. 2. Scheme of an LRETQ measurement at the cell surface. The labeling strategy is illustrated by the example of a transmembrane protein, class I MHC. The molecule is composed of 3 extracellular domains (α_1, α_2, α_3), a noncovalently associated light chain β2-microglobulin (β2m), and a transmembrane region ending in a short cytoplasmic tail. The heavy chains are labeled with fluorophore- (F) or quencher-conjugated (Q) Fab fragments of monoclonal antibodies (mAb).

Quenching of Fluorescein and Tb^{3+} Emission of MHC-Bound Antibodies by Nitroxide-Conjugated Antibodies at the Cell Surface: Theoretical and Strategic Background of LRETQ Measurements

VA-2 fibroblast cells were labeled with FITC- or CITC (+1 mM Tb^{3+})-conjugated KE-2 antibodies exploiting the luminescence enhancement upon chelation by CITC moiety (45). The labeling strategy of LRETQ experiments to analyze homoassociation of cell surface receptors on living cells is depicted on Figure 2 (for details see Materials and Methods section).

The quenching method is based on the Marcus theory of electron transfer (24,39,40). The rate of the quenching reaction between excited-state fluorophores and nitroxide quenchers via electron transfer mechanism is:

$$k_{et} = Ae^{-\frac{\Delta G^*}{RT}} \quad (1)$$

where A is primarily determined by electronic coupling of the donor and acceptor and denotes distance dependence of the electron transfer expressed by Eq. 3. ΔG^* is the free energy of activation which can be related to measurable quantities:

$$\Delta G^* = \frac{\lambda}{4}(1 + \Delta G^0)^2 \quad (2)$$

where λ is the reorganization (internal + solvent shell) energy, ΔG^0 is the free energy of the redox reaction.

Marcus (40) and Dexter (12) predicted the exponential distance-dependence of the electron transfer rate:

$$k_{et}(r) = k_0 e^{-\beta(R-R_0)} \quad (3)$$

where k_0 is the rate of electron transfer at the van der Waals contact distance (R_0), β is the steepness of the

distance-dependence that strongly depends on the medium separating the donor and acceptor by a distance of R. Thus, after a proper calibration, quenching by electron transfer (LRETQ) might have similar practical significance as an inter- or intramolecular ruler as the Förster-type resonance energy transfer.

The short range of effective interaction distances (max. 2.5 nm) makes the LRETQ approach uniquely powerful for detection of "physical association" between membrane proteins. Though a wide variety of quenching mechanisms are expected when fluorophores with different electronic structures, singlet energies, and redox potentials are used, only electron transfer was found to be the dominating mechanism in quenching by nitroxide radicals so far (25). This means that one can use a wide variety of fluorescent donors to nitroxide type acceptors for LRETQ measurements. On the cell surface the membrane proteins are selected by fluorophore-conjugated Fab fragments of monoclonal antibodies. The same membrane proteins are also labeled by quencher (nitroxide)-conjugated Fab fragments. In such a system one would expect quenching only between pairs of Fab-tagged membrane proteins located within a 2.0–2.5 nm center to center distance. Those Fab-labeled proteins which do not have protein molecules within a sphere of action of this size will contribute to the total fluorescence intensity with their own unquenched fluorescence intensities. This way this two-dimensional quenching is expected to show a dependence on the cell surface density of acceptor, similarly to the Förster-type resonance energy transfer (23). Any measurable quenching can be regarded as sign of partial association of electron donor and acceptor carrying membrane proteins due to their non-random colocalization. The extent of the quenching can be quantified either by displaying the data in a "two dimensional Stern-Volmer plot" or by displaying the fractional fluorescence intensity (F/F_0) against the fractional cell surface density (σ) of the quencher-carrying antibody (electron acceptor).

LRETQ Measurements on VA-2 Fibroblasts

Increasing the fractional density of quencher-conjugated antibodies, while keeping the total antibody concentration constant, a significant 30–40% quenching of both fluorescein and Tb^{3+} emission was observed at 1:1 luminophore to quencher molar ratio on VA2 cells (Fig. 3; see also Fig. 5). For fluorescein, the quenching data obtained by means of spectrofluorimetry and flow cytometry agreed well within the experimental error. This finding is in good accordance with FCET data obtained with FITC- and Texas-Red conjugated antibodies on the same cells (Table 1). The same extent of quenching with the two fluorophores with strongly different excited-state lifetimes (4 ns and 1.12 ms for FITC and Tb^{3+}, respectively) suggest that the extent of quenching is not determined preferentially by diffusional properties of the labeled species (44,45).

No significant quenching was observed on VA-2 cells when FITC-conjugated transferrin, bound to transferrin receptors, was used as electron donor and nitroxide-conjugated KE-2 antibodies were used as electron acceptor (Fig. 3).

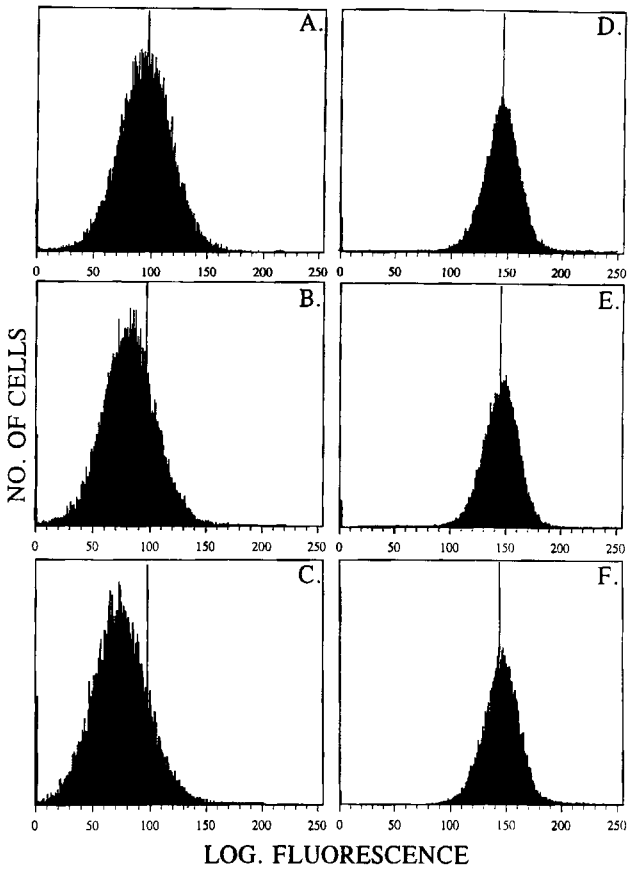

FIG. 3. Quenching of the fluorescence of FITC-KE2 antibodies and receptor-bound FITC-transferrin by EECP-KE2 antibodies at the surface of VA-2 fibroblast cells. The VA-2 fibroblast cells were labeled with increasing fractional density of quencher-labeled Fab (see Materials and Methods) at low temperature (0°C) and the fluorescence intensity histograms were recorded by flow cytometry. Histograms **A,B,** and **C** show the fluorescence of VA-2 cells labeled with FITC-KE2 at (σ_Q) quencher density values of 0, 0.2 and 0.5, respectively. Histograms **D,E,** and **F** represent the fluorescence of VA-2 cells labeled by FITC-transferrin at σ_Q values of 0, 0.2, and 0.5, respectively. The vertical lines represent the mean fluorescence of the unquenched samples.

LRETQ Between Heavy and Light Chains of Class I MHC Molecules

Fluorochrome conjugated monoclonal antibodies against the heavy chain and β2-microglobulin components of class I MHC molecules revealed a close proximity of these two antibody-binding sites using Förster-type resonance energy transfer technique (58).

Similarly a significant LRETQ was observed on JY cells (Fig. 4) when the light chain (β2-microglobulin) of class I MHC molecules was labeled with FITC-conjugated Fab fragments of BBM.1 monoclonal antibody and the heavy chains were labeled by nitroxide-conjugated Fab fragments of monoclonal antibodies KE-2 or BB7.2.

Table 1
Förster-Type Resonance Energy Transfer Between Fluorescein (D) and TEXAS RED (A) Labeled Antibodies Against Class I MHC Molecules on VA-2 and JY Cell Lines

Cell	Donor (determinant)	Acceptor	E (%)[a]
VA-2, human fibroblast	KE2 Fab (anti-HLA A,B,C)	KE2 Fab	7.8 (± 1.2)
	KE2 IgG	KE2 IgG	12.4 (± 1.7)
JY, human B lymphoblast	KE2 Fab	KE2 Fab	7.4 (± 2.1)
JY	TB-28 (anti-SIg)	KE2 Fab	< 1.0
JY	BB7.2 Fab (anti-HLA A2)	BB7.2 Fab	8.2 (± 2.2)
JY	W6/32 Fab (anti-HLA A,B,C)	W6/32 Fab	10.1 (± 2.7)

[a]E, the efficiency of the energy transfer, was determined from fluorescence cytograms recorded with donor-, acceptor-, and double-labeled cell populations on a Coulter Epics 752 flow cytometer. For each fluorescence histogram at least 20,000 cells were counted. Data were evaluated as described in the Materials and Methods. Values in parentheses indicate ± S.D.

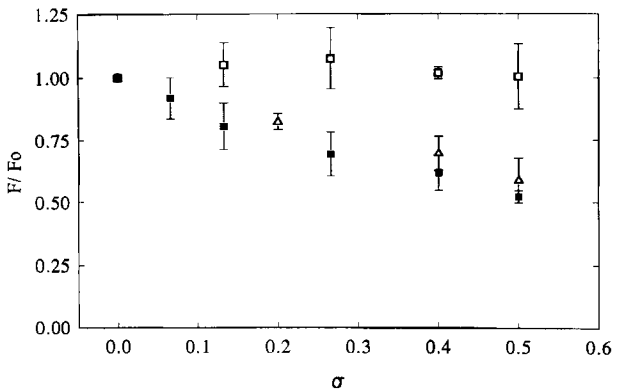

FIG. 4. Intra- and intermolecular quenching of the fluorescence from FITC-conjugated antibodies by EECP-KE2 on the surface of JY cells. The experiments were carried out as described in the legend to Figure 2 at different fractional quencher densities (σ_Q) displayed on the X axis. The fluorescence from FITC-KE2 (■), FITC-SIg (□), and FITC-BBM.1 (△) was quenched by EECP-KE2 at the surface of JY cells. The F/F_0 values were calculated from the means of histograms recorded by flow cytometry as described in Materials and Methods. F_0 and F values represent the means of fluorescence histograms measured in the absence and presence of quencher-conjugated antibodies (at different densities), respectively.

Homo- and Hetero-Association of Class I MHC Molecules on JY B Lymphoblast Cells

We tested the LRET quenching between fluorescein and EECP when both molecules labeled the heavy-chain (by conjugates of Fab fragment of KE-2 antibody) of the MHC molecule. There was a significant, about 45% quenching at 1:1 molar ratio between these two sites (Fig. 4). FRET data obtained with FITC- and Texas-Red-conjugated W6/32 antibodies are in good agreement with this finding. Antibodies directed against different epitopes of class I MHC molecule showed very similar energy transfer efficiencies (Table 1).

There was no detectable quenching, however, between FITC-conjugated anti-SIg antibody and nitroxide conjugated MHC-bound KE-2 antibody at the surface of JY cells (Fig. 4), also in good agreement with FRET data (Table 1).

Effect of Temperature, Cytochalasin D, and Fixation on the Efficiency of LRETQ at the Cell Surface

The LRET quenching data obtained on VA-2 cells between heavy chains of class I MHC molecules are displayed in Figure 5 in the form of Stern-Vomer plots. On these cells, no significant difference was found in quenching efficiency upon changing temperature of the measurements from approximately 4 to 37°C (data not shown). Similarly, no significant change in the Stern-Volmer plot was observed upon preincubation of the cells with 20 μM cytochalasin D or upon fixation of the cells by 2% paraformaldehyde before or after labeling, in ice (Fig. 5). We should note here that the paraformaldehyde itself reduced the quenching even in solution (data not shown), very likely via redox interaction with the nitroxide radicals. This was taken into account when evaluating quenching data of fixed cells. These results suggest that LRETQ reflects preexisting protein associations at the cell surface.

Quenching of Fluorescence of Membrane-Bound Proteins by Soluble Nitroxides: A Probe of Protein Conformation

Water-soluble nitroxides can be used as quenchers in free form, such as TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl) or the more polar TEMPOL (4-hydroxy-TEMPO), or as conjugates with monoclonal antibodies or their Fab fragments (TEMPO- or PROXYL-IgG, PROXYL-Fab). A Stern-Volmer analysis of quenching by nitroxide quenchers will yield information about changes in the degree of fluorophore-accessibility in labeled proteins, a potentially sensitive marker of conformation. Using *polar, non-permeable* TEMPO and PROXYL derivatives in studies with living cells is advantageous compared with ionic quenchers, such as KI, because nitroxides do not have ionic-strength effects and they are more effective quenchers of the most frequently used fluorophores in the visible wavelength range (e.g., fluorescein, rhodamine, eosin, etc.).

Figure 6 shows quenching of fluorescein fluorescence by water-soluble TEMPOL when the fluorescein is free in

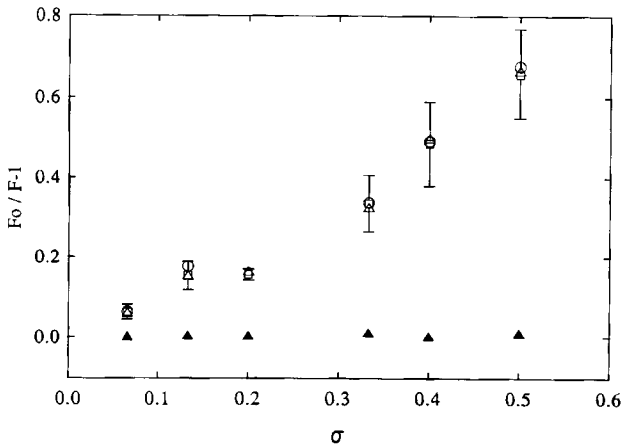

FIG. 5. Quenching of Tb^{3+} and fluorescein fluorescence on VA-2 cells by EECP-KE2. Stern-Volmer representation of quenching data obtained on VA-2 cells between Tb^{3+}/CITC- (○) or FITC-conjugated KE2 (△) and EECP-conjugated KE2. The effect of cytochalasin-D (20 μM) pretreatment of the cells on the quenching (□) is also displayed together with the effect of EECP-KE2 on the autofluorescence of the cells (▲).

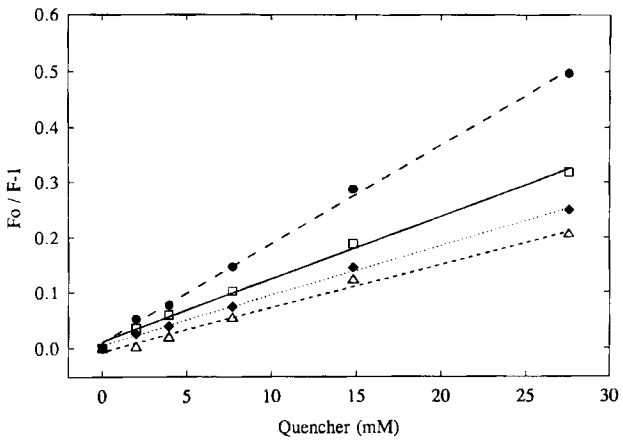

FIG. 6. Quenching of different forms of FITC probe by TEMPOL nitroxide. Stern-Volmer plots of the quenching of FITC fluorescence by TEMPOL in solution and at the cell surface. The free fluorescein (●), FITC-W6/32 IgG (□), FITC-W6/32 Fab (◆), and FITC-W6/32 IgG bound to the surface of JY lymphoma cells (△) were quenched with soluble TEMPOL in a spectrofluorimeter, at 37°C, in 10 mM Hepes-Hanks' buffer (+1 mM Ca^{2+}) at pH 7.4.

solution, coupled to antibody IgG or Fab, or coupled to antibody IgG bound to MHC I molecules at the cell surface. It can be seen that the protein-coupled fluorescein is less effectively quenched by TEMPOL than is free fluorescein, and that fluorescein-IgG bound to cells is the least quenched in the series. These differences certainly reflect steric hindrance of the approach of the quencher to the fluorophore. Potential changes in the magnitude of quenching, particularly for molecules bound to the cell surface, may reflect conformational changes of the molecules that in turn changes the accessibility of fluorophore to interaction with quencher.

DISCUSSION

The technique of quenching of fluorescence by paramagnetic probes (30,35) is widely used to reveal different aspects of molecular interactions in model and biological membranes (3,37). Several recent studies on semisynthetic and natural model systems (25,41,45) nicely confirmed the theoretical predictions by Dexter and Marcus (12,40) on the exponential distance-dependence of electron transfer reactions, such as the interaction of excited-state singlet and triplet probes with nitroxide radicals. The former experiments also demonstrated that an appropriate medium separating the electron donor and acceptor molecules may extend the range of effective ET distances up to 2.0–2.5 nm, possibly via tunneling mechanisms (1,29,41,48). This makes LRET potentially useful as an intra- or inter-molecular ruler in the distance range of 0.5–2.5 nm. Application of water-soluble nitroxides conjugated to monoclonal antibodies extends the application area of LRET to the cell surface phenomena such as homo- or hetero-associations of cell surface receptors. Conformational changes in the extracellular region of membrane-bound proteins can also be sensitively monitored by intramolecular or intermolecular LRETQ measurements.

Selection of the appropriate membrane protein for LRETQ measurements is usually done by tagging with Fab fragments of monoclonal antibodies. Application of Fab fragments vs. whole IgG molecules is recommended here for two reasons. Using Fab, one has a "monitor" with a size about one third of the IgG without significant loss in affinity to the antigenic site. Although, on the donor side this replacement results in a decreased level of fluorescence signal (a drawback), this is compensated by the advantage of avoiding such problems like triggering of cell activation by crosslinking the targeted cell surface receptors with bivalent IgG molecules (61).

Application of water-soluble nitroxides as electron-acceptor (quencher) has two obvious advantages compared to the fluorescent acceptors, such as tetramethylrhodamine or Texas-Red, applied most frequently in FRET experiments. Using water-soluble nitroxides the difficulties with non-specific binding and rapid internalization raised mostly by the hydrophobic, fluorescent acceptor molecules (Hochman and Edidin, unpublished results) are avoided. In addition, nitroxide labels are relatively small molecules, minimizing the hazard of structural perturbation during the antibody-conjugation. The nitroxide molecules are highly versatile quenchers since they are good electron acceptors for a wide variety of excited luminescent probes without the spectral-overlap limitations characteristic of the Förster-type donor-acceptor pairs.

LRETQ is also a good "molecular ruler," similar to the FRET, but with a significantly narrower range of effective distances. LRETQ is less sensitive to the relative orientation of donor and acceptor dipoles than the FRET. Therefore a combined application of the two distance-

dependent quenching methods may help to avoid the interpretation artifacts and provide a powerful tool in the analysis of homo- and hetero-associations of membrane proteins.

We worked out a strategy for LRETQ experiments on living cells to study homo-association of membrane proteins. This approach is based on the quenching of donor fluorescence by dark acceptors. Compared to the FRET measurements, this is an obvious drawback, because one should carefully test all the possible factors (other than a distance-dependent quenching interaction) resulting in a reduced donor fluorescence. Difficulties may arise from under- or over-estimation of protein concentrations during the labeling or from differences between binding affinities of unlabeled and differently conjugated antibodies. Thus a careful competition assay with unlabeled, fluorophore- and quencher-conjugated antibodies (or Fabs) is always essential. To avoid possible problems arising from insufficient accuracy of protein (antibody) concentration in the labeling mixture or from reduction of donor fluorescence upon binding unlabeled or quencher-labeled antibodies, introduction of an internal reference intensity is necessary, especially for steady-state measurements. This intensity can be measured relatively easily in an LRETQ system, when the cells have both fluorophore- and quencher-conjugated antibodies in the presence of 10 mM ascorbate. The latter treatment completely inactivates the quencher molecules (45), and the resulting intensity measured at such conditions can be regarded as a reference intensity (F_0) for the unquenched donor fluorescence. It should be noted that the excited-state lifetime of the donor is also reduced in the presence of nitroxide quenchers (Matko, unpublished observation), thus the method is not restricted to steady-state spectrofluorimetric, flow cytometric, or image microscopic measurements; it is potentially available for time-resolved measurements, as well.

LRETQ measurements were tested on biological systems where data about protein association were available from different approaches. LRETQ data were in good agreement with the results of FCET measurements performed on the same system at the same conditions, when self-association of class I MHC molecules was examined at the surface of JY human lymphoblasts (8,42). Other approaches, such as rotational diffusion measurements and FCET data with another donor acceptor pair (Bene et al., 1994 (Eur. J. Immunol., in press), also confirm our data. The efficiency of the LRETQ was surprisingly high, approaching the 1:1 donor to acceptor molar ratio on both JY lymphoblasts and VA-2 fibroblasts. This high quenching efficiency is very likely due to appearance of the electron donors-acceptor pairs within the quenching "sphere of action" with simultaneous existence of efficient through-bond tunneling pathways such as the polypeptide chain and carbohydrate moieties of the protein molecules (1,36).

The intramolecular LRETQ data between FITC and PROXYL moieties labeling a heavy- and light-chain epitope on class I MHC molecule are also in good agreement with earlier FCET data (58) and the X-ray crystallographic structure of the molecule (53). The lack of LRETQ for the protein pairs of transferrin receptors and class I MHC on VA-2 cells and SIg and class I MHC on JY cells confirms earlier findings (17,20) and also demonstrates the ability of the approach to monitor protein associations.

An important lesson can be drawn from these cell surface LRETQ measurements. In the case of *intra-molecular* quenching, the rate of LRETQ is preferentially determined by the separation distance between the donor and acceptor molecules (solvent effects are usually negligible in cell studies because the extracellular environment is always an isotonic, buffered bulk-water phase). However, in the case of *inter-molecular quenching*, the rate, in principle, can be determined by both the diffusion of the reactants and the separation distance. Thus, using singlet probes with relatively short (several ns) lifetimes is preferred in "mapping studies" since diffusion of the label-carrying large proteins in the highly viscous membrane will not have significant influence on the measured quenching efficiency. In the case of triplet probes, however, diffusion may also contribute to quenching depending on the excited-state lifetime and the relationship between energy levels of the emitter and the quencher. This feature, in principle, might be exploited in studying "short-range lateral diffusion" of membrane proteins at the cell surface.

Besides the experimental examples shown in the figures, the presence or lack of class I MHC clusters have been successfully detected on various other cells, as well in accordance with FCET or immunoprecipitation data (7,42,44).

Quenching of luminescence by free TEMPOL or by TEMPO- or PROXYL-IgG or Fab molecules all seem to be sensitive to changes in *conformational states* of membrane proteins. A nice example has been presented in a recent study on membrane-potential dependent conformational states of Band 3 protein in the human erythrocyte membrane (66), using quenching of triplet probes (eosin) attached to Band 3 by TEMPO. The present quenching experiments with fluorescein and TEMPOL also led to interesting results. The decreased quenching efficiency for FITC upon conjugation to IgG or Fab molecules is very likely due to masking effects by the protein binding site(s). A comparison of quenching efficiencies obtained with whole IgG and Fab fragments of the same antibody reflected a more compact/rigid structure of Fab compared to the flexible IgG_1 molecule. The quenching also revealed a further conformational change in the IgG molecule upon complexation with the antigen. This binding further decreased the accessibility of the FITC probes to TEMPO. A similar tendency in the magnitude of quenching constants was observed with the collisional quencher KI, though at lower quenching rates (45). These observations suggest that quenching by Tempol and other nitroxides is a sensitive kinetic marker of protein conformation with the additional advantage of eliminating ionic strength effects caused by the increasing concentration of ionic quenchers, such as KI.

In conclusion we can say that the LRETQ technique provides a wide range of applications to cell surface phenomena such as protein association or conformational changes. LRETQ studies can be performed on both cell suspensions (spectrofluorimetry, flow cytometry) or on single cells (image microscopy) in steady-state or time-resolved measurements. Compared with a similar "ruler" technique, the Förster-type resonance energy transfer, has the drawback of measuring only on the donor side. The short range of effective LRET distances allows an accurate detection of *physical association* between proteins. This approach, especially combined with FCET investigations, may provide us with a useful tool for studying receptor/antigen clustering in cellular physiology, since these two methods are the only techniques that probe protein patterns at the molecular level.

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