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Transport of a lysosomal membrane glycoprotein from the Golgi to endosomes and lysosomes via the cell surface in African trypanosomes

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SUMMARY

gp57/42 is a membrane glycoprotein localized in the *trans*-Golgi, flagellar pocket region of the cell surface, endosomes and lysosomes of bloodstream forms of *Trypanosoma brucei rhodesiense*. Pulse-chase immunoprecipitation experiments revealed that gp57/42 acquires a unique N-linked oligosaccharide recognized by the CB1 monoclonal antibody 20-30 minutes after protein synthesis, probably in the *trans*-Golgi. We refer to gp57/42 molecules that carry the CB1 eptiope as CB1-gp. Pulse labeled CB1-gp contained only one core protein, p57, when chase times were 30 minutes or less. As time of chase increased from 30 to 60 minutes, a new polypeptide, p42, appeared in N-glycanase-treated CB1 immunoprecipitates. Since p57 and p42 share 10 of 13 methionyl peptides, we conclude that p42 is a fragment of p57. Cleavage of p57 to p42 was not inhibited when cells were chased in two thiol protease inhibitors or in 3,4-diisocoumarin, but was inhibited by leupeptin. Cell surface biotinylation was used to determine if newly synthesized CB1-gp was transported from the Golgi to the surface. When cells were pulse labeled and chased for 30 minutes, as much as 40% of the radiolabeled CB1-gp could be biotinylated on the cell surface. The amount of CB1-gp that could be biotinylated decreased when chases were extended from 30 to 60 minutes, suggesting that pulse labeled CB1-gp left the surface. In contrast, pulse labeled variant surface glycoprotein molecules continued to accumulate on the surface where they could be biotinylated between 30 and 60 minutes of chase. Biotinylated CB1-gp derived from cells chased for 30 minutes contained p57 but no p42. However, when labeled cells were biotinylated after a 30 minute chase and then incubated another 30 minutes at 37°C, the biotinylated CB1-gp contained both p57 and p42. The p57 in biotinylated CB1-gp was not cleaved to p42 if the additional incubation was done at 4 or 12°C. This suggests that transport to a compartment where processing occurs and/or the processing enzymes are inhibited by low temperature. When surface biotinylation was done after a 60 minute chase, p42 was detected in biotinylated CB1-gp, suggesting that CB1-gp molecules had passed through the processing compartment and then appeared on the cell surface. Thus, a major portion of the newly synthesized CB1-gp is routed from the Golgi to endocytic compartments via the cell surface. In trypanosomes this process involves a unique surface domain, the flagellar pocket. Otherwise, this pathway of lysosomal membrane glycoprotein transport has been conserved in evolution from the trypanosomes to the vertebrates.

Key words: flagellar pocket, intracellular transport, lysosomal membrane glycoprotein, proteolytic processing, *Trypanosoma brucei rhodesiense*, endosome

INTRODUCTION

Vertebrate lysosomal membrane glycoproteins (LGPs) comprise a homologous group of integral membrane glycoproteins distributed throughout lysosomal and endosomal compartments (Chen et al., 1985; Fukuda, 1991; Furuno et al., 1989a,b; Granger et al., 1990; Lippincott-Schwartz and Fambrough, 1986). Studies using vertebrate cells suggest that newly synthesized LGPs can follow two different intracellular routes from the Golgi to endosomes and lysosomes. Some LGP molecules travel from the Golgi to the cell surface and subsequently to endosomes and lysosomes. This route can be readily demonstrated in transfected cells that are over producing LGP gene products (Harter and Mellman, 1992; Mathews et al., 1992; Williams and Fukuda, 1990) and in the highly polarized MDCK cell line; at least 70% of newly synthesized AC17 molecules appear on the basolateral surface of MDCK cells before being transported in endocytic vesicles to endosomes and lysosomes (Nabi et al., 1991). However, most studies have reported that only 0-5% of newly synthesized LGP molecules appear on the surface before entering endosomal compartments (Harter and Mellman, 1992; Carlsson and Fukuda, 1992; Lee et al., 1990), and that very little (Furuno et al., 1989a,b; Fambrough et al., 1988; Lippincott-Schwartz and Fambrough, 1987) or no (Chen et al., 1985; Granger et al., 1990; Harter and Mellman, 1992; Chen et al., 1988) steady state LGP can be detected on the cell surface. Because the surface pathway does not seem to be important in many cases, a direct pathway has been postulated to be the major route by which LGP120 (Harter and Mellman, 1992) and human LAMP molecules

(Carlsson and Fukuda, 1992) are transported from Golgi to endosomes. LGPs that use this direct route may also use the cell surface route as a minor pathway, and molecules transported to the surface may take much longer to reach lysosomes (Fukuda, 1991).

What determines whether an LGP appears on the surface is largely unknown. How much LGP a cell produces may be a factor. Thus, although LGP120 is not detectable on the surface of normal cells, a small amount of LGP120 does appear on the surface of heavily transfected cells that overproduce this protein (Harter and Mellman, 1992). However, increased expression of protein does not always correlate with increased cell surface expression; when HL60 cells differentiate, LAMP expression increases (Lee et al., 1990), but the proportion of LAMP molecules that use the cell surface route decreases (Carlsson and Fukuda, 1992). A tyrosine residue in the conserved C-terminal, cytoplasmic tail strongly influences how much of some LGPs accumulates on the surface of transfected cells and the subsequent targeting of these proteins to the lysosomes. Presumeably this region of the tail includes a motif that influences how efficiently LGP molecules are incorporated into the membranes of endocytic vesicles (Matthews et al., 1992; Williams and Fukuda, 1990).

Comparative studies of non-vertebrate LGPs may be useful in identifying the factors that control intracellular transport and targeting of LGPs, and in this paper we describe studies designed to determine if gp57/42, a LGP expressed by bloodstream forms (BF) of the human parasite *Trypanosoma brucei rhodesiense*, uses the cell surface route to reach early and late endocytic compartments. Our previous immunoelectron microscopy studies have suggested that newly synthesized gp57/42 molecules travel from the endoplasmic reticulum to the *trans*-Golgi where N-linked oligosaccharides are processed to generate a unique epitope that confers reactivity with the CB1 monoclonal antibody. We refer to gp57/42 molecules that have obtained the CB1 epitope as CB1-gp. Affinity-purified CB1-gp contains two closely related core proteins, p57 and p42. About 90% of the mature CB1-gp is localized in the membranes of coated endocytic vesicles, tubulovesicular endosomes, sometimes called collecting tubules or intermediate endosomes, and a large, perinuclear terminal endosome. The remaining 10% of CB1-gp is present is a specialized membrane domain on the cell surface called the flagellar pocket (FP; Brickman and Balber, 1993). The FP comprises only 0.4% of the surface area of BF (Coppens et al., 1987). Thus, although a low proportion of CB1-gp is present on the surface, the LGP is highly concentrated in the FP membrane.

One explanation for the presence of CB1-gp in the FP membrane is that BF use intracellular transport pathways similar to vertebrate cells and that newly synthesized CB1-gp molecules move from the Golgi to the FP en route to endocytic compartments. This idea is consistent with evidence that the FP is the only place where secretory vesicles add membrane proteins and endocytic vesicles remove membrane from the surface of BF (Balber, 1990; Webster and Russell, 1993). Alternatively, CB1-gp could reach endocytic compartments by a direct pathway from the *trans*-Golgi without first appearing on the cell surface. Variant surface glycoprotein (VSG), an abundant glycoprotein that forms a coat on most of the BF surface membrane (Balber, 1990; Cross, 1990), is believed to recycle rapidly to the surface after being endocytosed (Seyfang et al., 1990; Webster and Grab, 1988). CB1-gp molecules could reach the FP by using the mechanisms that return VSG (Seyfang et al., 1990; Webster and Grab, 1988; Duszenko and Seyfang, 1994), and perhaps some other trypanosome membrane proteins (Coppens et al., 1993), to the surface. LEP100 (Fambrough et al., 1988; Lippincott-Schwartz and Fambrough, 1987), LGP107 (Furuno et al., 1989a,b) and other vertebrate LGPs (Harter and Mellman, 1992) may also reach the surface by recycling from an internal compartment.

In this paper we present the results of pulse-chase immunoprecipitation and cell surface biotinylation experiments designed to study the post-translational processing and transport of CB1-gp. We demonstrate that CB1-gp travels from the Golgi to endocytic compartments via the cell surface and that this pathway of intracellular transport has therefore been conserved in evolution from the trypanosomes through the vertebrates.

MATERIALS AND METHODS

Parasites

The derivation, maintenance and isolation of BF of the DuTat 1.1 clone of the Wellcome CT strain of *Trypanosoma brucei rhodesiense* has been described previously (Frommel and Balber, 1987).

[^{35}S]methionine labeling and immunoprecipitation of purified CB1-gp

BF were isolated in methionine-deficient minimal essential medium (Brickman and Balber, 1993), incubated at 6×10^7 cells/ml for one hour and then labeled with [^{35}S]methionine (250 μCi/ml) at 3×10^8 cells/ml for five minutes. The cells were then washed once and resuspended at 6×10^7 cells/ml in HMI-9 tissue culture medium prepared as described by Carruthers and Cross (1992) except that 1% (v/v) normal goat serum was used in place of heat-inactivated fetal bovine serum. This medium is refered to as chase medium (CM). Cells were chased under conditions described in the text.

Following the chase, the cells were washed and ricin-binding glycoproteins (RBGPs) were extracted by the procedure of Brickman and Balber (1993) with two important changes. First, Chaps was substituted for Triton X-100 in all steps; this provided much more complete extraction of RBGPs from cytoskeletons and better yields of CB1-gp. Second, in lectin affinity chromatography steps, a pH 4.0 wash was added prior to elution of CB1-gp at pH 2.0. This step greatly reduced non-specific binding during lectin affinity purifications. Parasites were extracted in 5% (w/v) Chaps in the extraction buffer, and the soluble extracts were incubated with ricin-agarose beads (EY Laboratories, CA) overnight at 4°C. Beads were washed three times with PBS-0.5% Chaps, and eluted sequentially with 0.1 M acetic acid in PBS-0.5% Chaps, pH 4.0, and then with 0.1 M phosphoric acid in PBS-0.5% Chaps, pH 2.0.

CB1-gp was immunopreciptated from neutralized pH 2.0 eluates. Eluates were incubated with Protein A/Sepharose beads for two hours to preclear any nonspecific binding, incubated with CB1 ascites overnight at 4°C, and then immunoprecipitated with rabbit anti-mouse IgG+IgM (H+L)- (Jackson Laboratories, ME) coated Protein A beads for four hours at 4°C. The beads were washed four times with PBS-0.5% Chaps, then eluted with 0.1 M phosphoric acid in PBS-0.5% Chaps at pH 2.0. The eluates were neutralized, ethanol precipitated with glycogen carrier, lyophilized, and stored at −70°C.

In some experiments, cells were preincubated for one hour, methionine-labeled and chased in the presence of 1 μg/ml tunicamycin (Calbiochem, CA), 40 μM E64 and 4 μM cystatin (Sigma), 500 μM leupeptin, or 10 μM 3,4-DCI (Boehringer Mannheim, IL).

Biotinylation of surface proteins

BF were preincubated, [^{35}S]methionine-labeled at 1×10^9 cells/ml, washed and chased under various conditions described in the text. The cells were washed three times at 4°C with phosphate buffered saline, pH 7.4, containing 1% (w/v) glucose, resuspended at 6×10^7 cells/ml, and incubated in the presence of 200 μg/ml sulfo-NHS-biotin (Pierce, IL) for 30 minutes at 4°C. The cells were then washed twice in CM before being processed as described in the text.

Biotinylated CB1-gp was prepared from labeled BF by affinity chromatography. RBGPs were prepared from biotinylated cells as described above. The pH 2.0 eluates were neutralized and applied to a CB1-agarose affinity column at 4°C. Samples were recycled over the column three times, the unbound material was collected, the bound material was eluted with 0.1 M phosphoric acid, pH 2.0, neutralized, ethanol precipitated with glycogen as a carrier and lyophilized. The lyophilized pellets were then dissolved in PBS-0.5% Chaps and incubated overnight at 4°C with avidin-agarose beads (Pierce, IL). The beads were washed four times with PBS-0.5% Chaps, and bound material was eluted by boiling the beads in the presence of PBS-0.5% Chaps containing 2% (w/v) SDS for five minutes. This was repeated a total of three times. The eluates were then pooled and ethanol precipitated with glycogen, lyophilized and stored at −70°C.

Lyophilized pellets were dissolved in Endoglycosidase F/N-glycosidase (Endo F/N) buffer and digested with Endo F/N (Brickman and Balber, 1993). Undigested controls were incubated under identical conditions without enzyme.

SDS-PAGE

Samples were boiled in 10× SDS-PAGE sample buffer for five minutes, separated electrophoretically on a 10% SDS-PAGE slab gel, fluorographed and exposed to film or to a phosphoimager screen for densitometric analysis. Densitometry was performed using a Molecular Dynamics Phosphor Imager and Image Quant, version 3.3, software. Volume measurements given in the text are directly proportional to band intensity.

RESULTS

Kinetics of post-translational acquisition of the CB1 epitope

Our previous immunoelectron microscopy studies suggested that the CB1 epitope is acquired in the *trans*-Golgi (Brickman and Balber, 1993). If this is true, CB1-gp should not be immunoprecipitated by the CB1 monoclonal antibody immediately after synthesis of the core protein, and there would be a lag period after pulse labeling when no CB1-gp could be immunoprecipitated. To test this prediction, BF were pulse labeled with radiomethionine for five minutes, then chased for as long as three hours before immunoprecipitation with CB1. Fig. 1 shows that very little radiolabeled material was immunoprecipitated from cell extracts at 0 and 10 minutes after pulse labeling, but that the amount of radioactivity immunoprecipitated increased sharply between 20 and 30 minutes, and reached a maximum by 60 minutes of chase. The half-time for the increase was about 25 minutes. The amount of radioactivity immunoprecipitated with CB1 remained high, but decreased slightly during the next 2 hours of chase. Similar kinetics, including the decrease in radioactivity during extended chases, was observed in each of five experiments.

The proteins that were present in these immunoprecipitates were analyzed by SDS-PAGE and fluorography. Fig. 2 shows that a small amount of radioactivity was associated with a

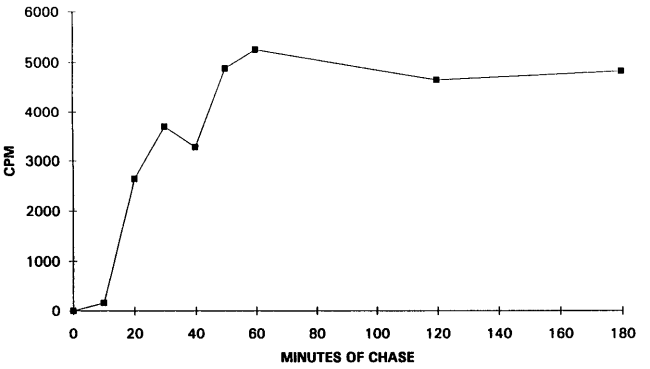

Fig. 1. Total amount of [^{35}S]methionine incorporated in immunoprecipitates of CB1-gp. BF were pulse labeled and chased at 37°C for the time shown on the abscissa. The ordinate represents the amount of [^{35}S]methionine incorporated in immunoprecipitates of CB1-gp derived from 1×10^8 cells.

single band or doublet migrating at a relative molecular mass M_r=22,000. This 22 kDa component (p22) was detectable as early as 10 minutes after labeling and increased slightly in amount during the first hour of chase (Fig. 2). However, more that 95% of the radioactivity in immunoprecipitates prepared during the first hour of chase migrated with CB1-gp at M_r=180,000 (Fig. 3). Very little radioactivity was recovered in this CB1-gp band 0-10 minutes after labeling, but the amount of radiolabeled CB1-gp increased rapidly between 20 and 60 minutes of chase (Figs 2 and 3) as expected if addition of the CB1 epitope is a late post-translational processing event that occurs in the Golgi. Subsequent proteolysis of CB1-gp during prolonged chases is discussed below.

Since N-linked oligosaccharides are important components of the CB1 epitope (Brickman and Balber, 1993), we expected that tunicamycin added to the preincubation, label and chase steps would inhibit the appearance of mature CB1-gp. Fig. 4 shows that tunicamycin had the expected effect. Densitometric analysis revealed that tunicamycin reduced the amount of radiolabeled CB1-gp immunoprecipitated from cells chased for 30 minutes by 64% in the experiment shown. The overall rate of protein synthesis measured by radiomethionine incorporation was inhibited by only 5%.

Fig. 4 also shows that very little CB1-gp was immunoprecipitated from BFs chased at 12°C instead of 37°C for 1.5 hours. Densitometric analysis showed that the recovery of CB1-gp was inhibited by 82% relative to cells chased at 37°C. Chasing at low temperature inhibits intracellular transport of newly synthesized proteins in trypansomes (Bangs et al., 1986) and other eucaryotes (Gruenberg and Howell, 1989), but could also inhibit enzymes mediating post-translational modifications.

CB1-GP undergoes proteolysis

Newly synthesized CB1-gp appeared as a very discrete, homogeneous band (Fig. 2). However, after 2 and 3 hours of chase, CB1-gp appeared as a more heterodisperse, broad band on an SDS-PAGE gel (Fig. 2). During the second and third hour of chase, the total amount of [^{35}S]methionine in immunoprecipitates remained relatively constant (Fig. 1), but the proportion of counts associated with CB1-gp decreased (Fig. 3). New, more rapidly migrating radiolabeled components of M_r=60,000

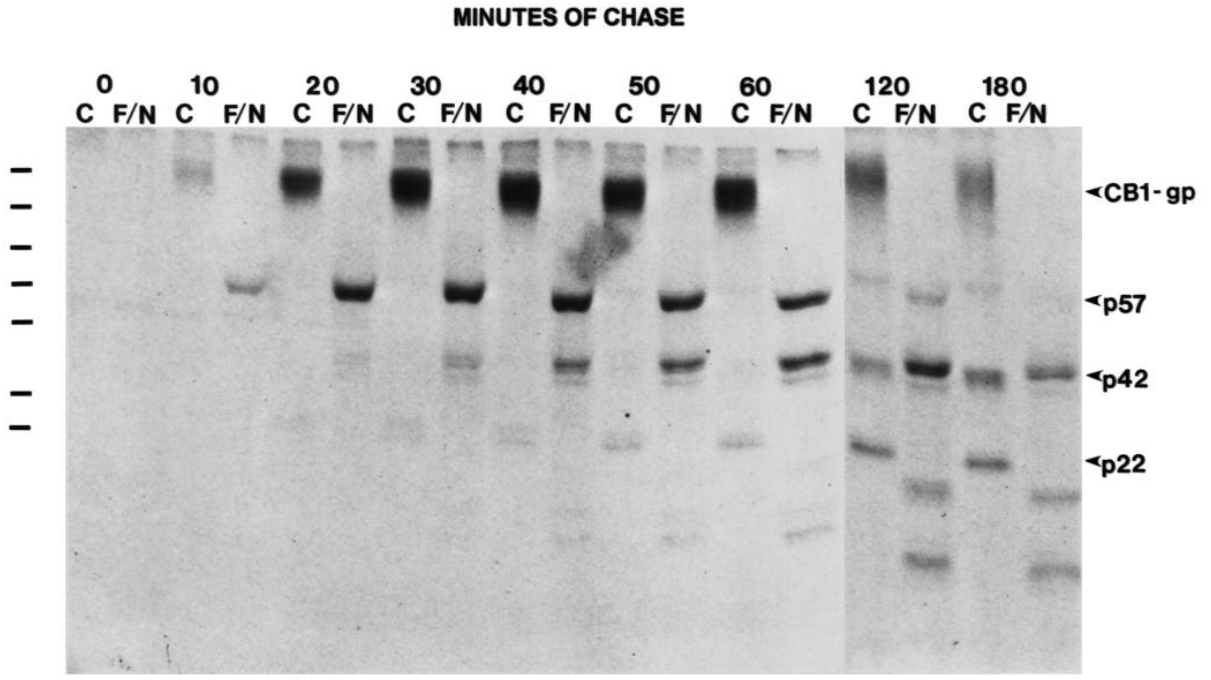

Fig. 2. Pulse-chase immunoprecipitation analysis of CB1-gp. BF were pulse labeled and chased at 37°C for the times shown. CB1-gp immunoprecipitates were prepared from 5×10^7 BF. The immunoprecipitates were either digested with EndoF/N (F/N) or left untreated (C). Labeled polypeptides were resolved on a 10% SDS-PAGE gel and fluorographed. Bars to the left of the gel represent molecular mass standards of 180, 116, 84, 58, 48, 36, and 26 kDa.

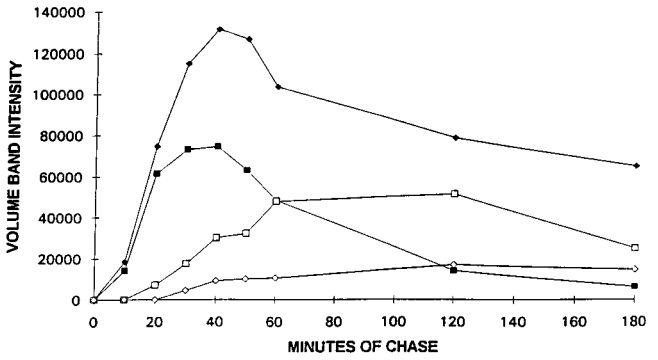

Fig. 3. Densitometric analysis of experiment shown in Fig. 2. The ordinate represents band image volume, which directly correlates with the intensity of the corresponding band. The abscissa shows time of chase. Data from CB1-gp (◆) and p22 (◇) bands are derived from undigested immunoprecipitates; data from p57 (■) and p42 (□), from EndoF/N-treated immunoprecipitates.

and 40,000, which are most likely proteolytic fragments of CB1-gp, appeared in immunoprecipitates 2-3 hours after labeling.

To determine if the apparent proteolysis of the CB1-gp over time resulted from cell death or non-specific proteolysis, we measured the electrophoretic mobility of VSG and the other pulse labeled polypeptides in these cell extracts. Even after 3 hours of chase, no evidence for proteolysis of any radiolabeled component was seen. None of the proteolytic fragments of DuTat 1.1 VSG that appear when cells are stressed (Frommel et al., 1988) was detected (data not shown). Thus, CB1-gp appears to be specifically cleaved during chase.

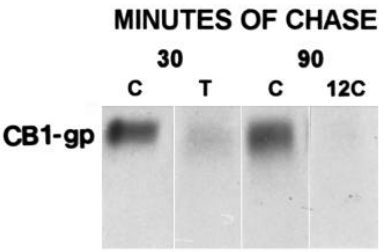

Fig. 4. Pulse-chase immunoprecipitation of CB1-gp at 12°C or in the presence of tunicamycin. BF were preincubated, pulse labeled, and chased for 30 minutes at 37°C with (T) or without (C) 1 μg/ml tunicamycin. Other samples of BF were preincubated and pulse labeled at 37°C and then chased for 90 minutes at either 37°C (C) or at 12°C (12C). CB1-gp was immunoprecipitated and analyzed on a 10% SDS-PAGE gel (5×10^7 cell equivalents/lane) and fluorographed.

Kinetics of p57 and p42 appearance in CB1-gp immunoprecipitates

We immunoprecipitated CB1-gp with CB1 and then digested the immunoprecipitate with EndoF/N to analyze the core proteins in mature glycoprotein at various times after pulse labeling. In our previous work (Brickman and Balber 1993), we demonstrated that CB1-gp that had been immunoprecipitated after 45 minutes of continuous labeling with radiomethionine yielded two proteins, p57 and p42, after EndoF/N digestion. Figs 2 and 3 show that p57 was the major protein component detected in CB1-gp immunoprecipitates during the first 30 minutes of chase. p57, but not p42, was present in these immunoprecipitates after 10 minutes of chase, the earliest time that we were able to detect mature CB1-gp. p42 was first

Table 1. The effects of protease inhibitors on the cleavage of p57 into p42

	Experiment 1			Experiment 2		Experiment 3	
	None	Leu	DCI	None	Leu	None	E/C
p57	64.0	83.0	55.0	54.9	70.3	65.0	62.0
p42	36.0	17.0	45.0	45.1	29.7	35.0	38.0

BF were preincubated, pulse-labeled and then chased for 90 minutes at 37°C in the presence of 500 μM leupeptin (Leu); 10 μM 3,4-diisocoumarin (DCI); 40 μM E-64 and 4 μM cystatin (E/C); or no inhibitor (None). CB1-gp was then immunoprecipitated, digested with EndoF/N, resolved on a 10% SDS-PAGE gel, fluorographed, and analyzed by densitometry. The values given are the percentage of the total densitometric volume units in CB1-gp associated with p57 and p42.

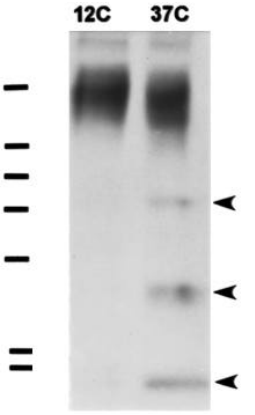

Fig. 5. Proteolysis of CB1-gp during prolonged chase at different temperatures. BF were pulse labeled, chased at 37°C for 30 minutes and then chased for 90 minutes at either 12°C (12C) or 37°C (37C). CB1-gp was immunoprecipitated and analyzed on a 10% SDS-PAGE gel (1×10^8 cell equivalents/lane), and fluorographed. Mass standards (bars at left) are as described in Fig. 2. Arrows point to the 60, 40 and 22 kDa fragments in material chased at 37°C.

detected after 30 minutes of chase, and its signal was relatively weak. As the time of chase increased, the intensity of p42 increased, and the intensity of p57 decreased. At 60 minutes of chase p57 and p42 were present in virtually equal amounts. After two hours of chase, very little p57 was detected, and p42 was the major EndoF/N product. We showed previously that p57 and p42 share 10 of 13 methionyl peptides (Brickman and Balber, 1993). Taken together, all these data suggest that p42 is derived from p57.

A leupeptin-sensitive protease mediates cleavage of p57 to p42

In order to determine what proteases may mediate the cleavage of p57 into p42, the preincubation, label and chase steps were performed in the presence of various protease inhibitors (North et al., 1990). Immunoprecipitated CB1-gp was digested with EndoF/N to release p57 and p42, and the amount of radioactivity in each band was determined by densitometry on the phosphoimager. Representative results are shown in Table 1. The cysteine protease inhibitors E64 and cystatin and the serine protease inhibitor 3,4-DCI did not inhibit cleavage of p57 to p42, but leupeptin reduced the proportion of p42 in CB1-gp immunoprecipitates about two-fold.

Chasing at 12°C inhibits processing of CB1-gp

We did experiments to determine whether chasing a homogeneous population of newly synthesized CB1-gp at 12°C altered proteolytic processing of CB1-gp. Pulse labeled BF were chased at 37°C for 30 minutes to permit addition of the CB1 epitope to CB1-gp, and then were incubated at either 12°C or 37°C for 1.5 hours to permit proteolytic processing to occur. Fig. 5 shows that CB1-gp was heterodisperse, and proteolytic fragments were present in immunoprecipitates derived from cells incubated at 37°C for the entire 2 hours. Material from cells chased at 12°C was more homogeneous on an SDS-PAGE gel, and the proteolytic fragments of CB1-gp that were present in cells chased at 37°C were not seen in material from cells chased at 12°C. Immunoprecipitates from this experiment were then digested with EndoF/N to determine whether the cleavage of p57 into p42 is also inhibited by a 12°C incubation. Densitometric analysis showed that immunoprecipitates derived from cells chased at 37°C had 3.9-fold more p42 than cells chased at 12°C (Table 2, experiment 1).

Table 2. Densitometric analysis of core proteins in Endo F/N-treated CB1-gp immunoprecipitates

	Experiment 1		Experiment 2			
	Total CB1-GP		Avidin bound		Avidin unbound	
°C	p57	p42	p57	p42	p57	p42
12	88.4	11.3	98.3	1.7	98.0	2.0
37	56.0	44.0	88.0	12.0	86.6	13.4

In both experiments, BF were pulse labeled with radiomethionine and chased for 30 minutes at 37°C. In Experiment 1, BF were incubated for 90 minutes at the temperatures shown. CB1-gp was then immunoprecipitated and EndoF/N treated. Core proteins were resolved by SDS-PAGE, and p57 and p42 were measured by densitometry. In Experiment 2, BF were then biotinylated and subsequently incubated at the temperatures shown for an additional 30 minutes. CB1-gp was then immunopreciptated, fractionated on avidin-agarose into biotinylated (avidin-bound) and non-biotinylated (avidin unbound) fractions, and digested with EndoF/N prior to electrophoretic analysis. The values given are the percentage of the total densitometric volume units in the immunoprecipitates associated with p57 or p42. These values have been corrected by subtracting the amount of p42 present after the initial 37°C chase; consequently the values show the change in the percentage of p42 occuring during incubation at the different temperatures.

Newly synthesized CB1-gp appears on the cell surface

In order to determine whether newly synthesized CB1-gp is transported from the *trans*-Golgi directly to proteolytic processing compartments or is first transported to the cell surface, we used sulfo-NHS-biotin to label surface proteins on BF. Because VSG is present in such high concentration, the vast majority of labeling reagent is typically associated with VSG in such experiments (reviewed by Overath et al., 1994). In preliminary experiments (not shown), we established, first, that the conditions described in Materials and Methods give maximum labeling of VSG and, second, that under these conditions biotin labeling detectable by cryoimmunoelectron microscopy with gold-conjugated anti-biotin antibody was restricted to the surface of BF; no internal labeling was seen.

Fig. 6A shows that a significant proportion of newly synthesized CB1-gp reached the cell surface where it became accessible to exogenous sulfo-NHS-biotin during a 60 minute chase. Densitometric analysis of the experiment in Fig. 6A showed that the proportion of CB1-gp recovered on avidin beads was 8% of the total amount of CB1-gp immunoprecipitated when BF were biotinylated after a 15 minute chase and

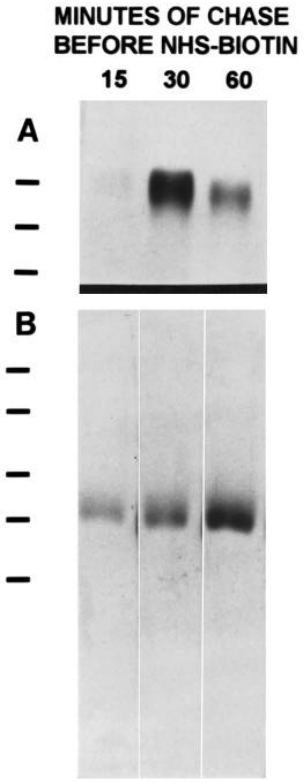

Fig. 6. Pulse-chase, surface biotinylation analysis of CB1-gp and VSG transport to the surface of BF. (A) Pulse labeled CB1-gp appears on the surface of BF. Pulse labeled BF were surface biotinylated after 15, 30 or 60 minutes of chase at 37°C. Chaps-soluble material was incubated with ricin-agarose. The material that bound to ricin-agarose was eluted, and CB1-gp was immunoprecipitated and then fractionated with avidin-agarose into avidin-bound (biotinylated, cell surface) and unbound fractions. The avidin-bound fraction was then analyzed on a 10% SDS-PAGE gel (2×10^8 cell equivalents/lane), and fluorographed. Mass standards on left are 180, 116, and 84 kDa. (B) Pulse labeled VSG accumulates progressively on the cell surface during chase. Chaps-soluble material from the experiment described above that did not bind to ricin-agarose was fractionated on avidin-agarose. The figure shows material eluted from the avidin beads. Samples were separated on a 10% SDS-PAGE gel (1×10^8 cell equivalents/lane) and fluorographed. Mass standards are 180, 116, 84, 58 and 48 kDa.

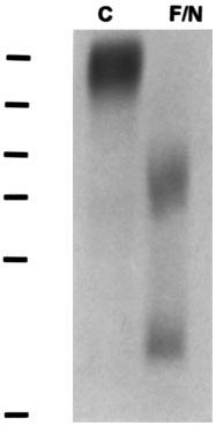

Fig. 7. CB1-gp biotinylated after prolonged chase contains p42. BF were pulse labeled, chased for 60 minutes at 37°C and then surface biotinylated. CB1-gp was immunoprecipitated, then fractionated with avidin-agarose. CB1-gp that bound to avidin was then digested with EndoF/N (F/N) or left undigested (C), analyzed on a 10% SDS-PAGE gel (6000 cpm/lane), and fluorographed. Mass standards on left are 180, 116, 84, 58, 48 and 36 kDa.

Table 3. Densitometric analysis of core proteins in cell surface CB1-gp derived from BF biotinylated at different times during chase

37°C Chase for	p57	p42
30 Minutes	94.6	5.4
60 Minutes	61.3	38.7

BF were pulse labeled with radiomethionine and chased for 30 or for 60 minutes at 37°C before being biotinylated. CB1-gp was then immunoprecipitated, fractionated on avidin-agarose into biotinylated and non-biotinylated fractions, EndoF/N digested and analyzed by electrophoresis and densitometry. The values given are the percentage of the total densitometric volume units in the biotinylated CB1-gp fraction associated with p57 or p42.

40% of the total when biotinylation was done after a 30 minute chase. Thus, the proportion of newly synthesized CB1-gp that reached the surface increased about 12-fold between 15 and 30 minutes of chase. Since more than half of pulse labeled CB1-gp molecules gain the CB1 epitope in the Golgi (Fig. 1) during the 15-30 minute chase period, and at least 40% of all pulse labeled, CB1-bearing molecules can be biotinylated at 30 minutes, transit from the Golgi to the surface is highly efficient. Between 30 and 60 minutes of chase, the proportion of newly made CB1-gp molecules that could be biotinylated on the surface decreased from 40% to 22%, suggesting that a significant number of CB1-gp molecules moved off the surface during this time period.

These results with the endosomal/lysosomal protein CB1-gp are in marked contrast to those obtained with the surface protein VSG in the same experiment. Fig. 6B shows that the amount of pulse labeled VSG recovered on avidin beads increased when the interval between pulse labeling and biotinylation increased from 15 to 30 minutes, but, unlike CB1-gp, continued to increase between 30 and 60 minutes. Pulse labeled VSG reaches the BF surface of other clones with a half-time of approximately 15 minutes (Bangs et al., 1986; Ferguson et al., 1986).

p57 is cleaved to p42 after transport to the surface

The data in Fig. 6 indicate that a significant portion of newly synthesized CB1-gp travels to the cell surface within 30 minutes of obtaining the CB1 epitope and then leaves the surface. In order to determine if the cleavage of p57 into p42 takes place before or after CB1-gp arrives at the surface, BF were pulse labeled and chased for 30 minutes at 37°C. This allowed newly synthesized CB1-gp to reach the cell surface. The cells were then surface biotinylated for 30 minutes at 4°C. After biotinylation, the BF were incubated at either 4, 12 or 37°C for an additional 30 minutes, to allow further transport and processing of labeled CB1-gp. The cells were then immunoprecipitated with CB1, and fractionated into biotinylated and non-biotinylated fractions with avidin-agarose and digested with EndoF/N. Samples of biotinylated CB1-gp derived from cells chased at 37°C had 6.8 times more p42 than samples from cells chased at 12°C (Table 2). These results show that very few CB1-gp that reach the surface within 30 minutes after pulse labeling contain p42 and that p57 is cleaved to p42 after these CB1-gp molecules appear on the cell surface.

Some p42 appears on the cell surface after prolonged chases

BF were pulse labeled, chased for 60 minutes at 37°C, and then were surface biotinylated. CB1-gp was immunoprecipitated with CB1 immediately following biotinylation and fractionated with avidin-agarose beads. Fig. 7 shows that both p57 and p42 were present in biotinylated CB1-gp isolated 60 minutes after labeling; about 38.7% of the immunoprecipitated material was p42. This contrasts sharply with experiments such as that shown in Table 3 when cells were biotinylated after a 30 minute chase; in this case, only 5.4% of the immunoprecipitated material was p42. This suggests that CB1-gp molecules can appear on the cell surface after p57 is cleaved to p42.

DISCUSSION

We believe our data provides the first evidence in a non-vertebrate system showing that newly synthesized LGPs travel from the *trans*-Golgi to the cell surface and then to endocytic compartments. In what follows we discuss data related to each step in this process.

The CB1 monoclonal antibody was first able to immunoprecipitate significant amounts of CB1-gp 30 minutes after pulse labeling BF with [^{35}S]methionine. The time lag between synthesis of CB1-gp and our ability to detect it with the CB1 monoclonal antibody probably reflects the time required for newly synthesized CB1-gp to reach the *trans*-Golgi, for CB1 does not react with endoplasmic reticulum or *cis*-Golgi elements (Brickman and Balber, 1993). Late Golgi processing events also take place in the biosynthesis of VSG (Bangs et al., 1986) and soluble acid phosphatase of closely related *Leishmania* parasites (Bates et al., 1990; Schell et al., 1990). In our previous work, we showed that galactose and lactose inhibited binding of the CB1 monoclonal antibody to CB1-gp and that CB1-gp binds strongly to ricin, a lectin that reacts strongly with terminal beta 1,4-galactosyl residues (Lis and Sharon, 1986). These observations suggest that galactose-containing sugars are important in the CB1 epitope. However, the CB1 epitope is not simply a terminal galactosyl group as CB1 does not bind to other ricin-binding glycoproteins (M. J. Brickman and A. E. Balber, unpublished data). Several beta galactosyltransferases have been reported to partition with Golgi fractions (Grab et al., 1984). The intracellular location of alpha galactosyltransferases is unknown (Pingel and Duszenko, 1992), but addition of alpha-galactosyl residues to the VSG glycolipid anchor is a late, presumably Golgi or post-Golgi, processing step (Bangs et al., 1988). Since we do not detect CB1-gp in the endoplasmic reticulum or the *cis*-Golgi (Brickman and Balber, 1993), the unique glycosyltransferase or other sugar modifying enzymes that create the CB1 epitope probably reside in the *trans*-Golgi.

We reported previously (Brickman and Balber, 1993) that p57 and p42 are closely related based on their peptide maps after V8 protease digestion. However, we could not determine if p57 and p42 were the products of one gene or of two closely related genes. The pulse-chase experiments in this report suggest that p57 is in fact the protein core of CB1-gp and that p42 is a major proteolytic fragment of p57. In pulse-chase, immunoprecipitation experiments, p57 was detected after 10 minutes of chase, the first time we were able to detect any CB1-gp. p42, on the other hand, was not detected until after 30 minutes of chase, and the amount we detected was very low. As time of chase increased, the amount of p57 decreased while the amount of p42 increased, while the overall amount of incorporated label remained relatively constant. This indicates that a precursor/product relationship exists between p57 and p42.

Our inhibitor studies indicate that specific leupeptin-sensitive, E64-, cystatin-, and 3,4-DCI-resistant proteases mediate this conversion. None of the proteases that have been purified from these parasites have precisely these inhibition characteristics (Kornblatt et al., 1992; Mbawa et al., 1991; Pamer et al., 1990; Steiger et al., 1980). Protease activities that respond to inhibitors in the same way as the p57 cleaving enzyme have been identified in lysates of BF (Huet et al., 1992; Lonsdale-Eccles and Grab, 1987; Robertson et al., 1990) but have not been purified. The functional significance of the p57 to p42 cleavage is unknown. Cleavage of p57 could be the first step in degradation of CB1-gp. Grab et al. (1993) recently showed that incubating BF in protease inhibitors increased the amount of transferrin-binding protein detectable in lysosomes and suggested that this surface protein is degraded after endocytosis. Alternatively, the conversion of p57 to p42 could activate or modulate novel CB1-gp functions when the protein reaches endosomal compartments.

More extensive proteolysis of CB1-gp is likely to be responsible for the spreading of the CB1-gp band in SDS-PAGE gels and the appearance of other rapidly migrating bands in the CB1 immunoprecipitates derived from BF chased for two and three hours. Proteolysis of CB1-gp explains the high degree of heterogeneity that we observed previously in immunoblots of whole cell extracts and in immunoprecipitates of continuously radiolabeled cells (Brickman and Balber, 1993).

Some other LGPs appear to be much more stable to proteolysis than CB1-gp. We detected degradation of CB1-gp after 2 hours of chase. Pulse labeled LEP100 was not proteolyzed detectably during 35 hours of chase (Lippincott-Schwartz and Fambrough, 1986). LGP120 was almost entirely protected from protease treatment in intact membranes, but was readily digested in the presence of detergent (Howe et al., 1988). Why the trypanosome protein is relatively sensitive to degradation is unknown. Differences in sugar structure could be involved, for long, poly-*N*-acetyllactosaminyl oligosaccharides are believed to play a role in protecting vetetebrate LGPs from proteases (Lee et al., 1990; Carlsson and Fukuda, 1990). Only short N-linked, poly-*N*-acetyllactosaminyl sugars have been detected in VSG (Zamze et al., 1991).

One rapidly migrating band, p22, does not appear to be derived from CB1-gp by proteolysis. p22 was specifically immunoprecipitated with CB1-gp after 30 minutes of chase. Very little p42 had been generated from p42 at this time. p22 does not immunoblot with the CB1 monoclonal antibody. This suggests that p22 does not possess the CB1 epitope and is coprecipitated with CB1-gp because it is associated with CB1-gp. A 22 kDa protein was also coprecipitated with gp65, a LGP associated with intermediate endosomes of *Trypanosoma vivax* (Burleigh et al., 1993). However, p22 cross-reacts with a monoclonal antibody to a peptide epitope of gp65 and was therefore considered to be a proteolytic fragment of gp65.

A significant portion of newly synthesized CB1-gp molecules appear on the cell surface where they can be biotinylated before they are proteolytically processed. CB1-gp biotinylation efficiency appears to be greatest after 30 minutes of chase. As much as 40% of pulse labeled CB1-gp immunoprecipitated from BF biotinylated after a 30 minute chase could be recovered on avidin beads. Less CB1-gp could be biotinylated on the surface following 15 or 60 minute chases. This suggests that the Golgi to cell surface route is an important pathway of CB1-gp protein transport.

Between 30 and 60 minutes of chase, the amount of pulse labeled CB1-gp that can be biotinylated on the cell surface decreases, the amount of p57 present in immunoprecipitates decreases, and the amount of p42 increases. The disappearance and processing of CB1-gp from the surface during this period contrasts with the continued accumulation of non-proteolyzed VSG on the surface during this period. We propose that these observations collectively reflect specific removal of CB1-gp

from the cell surface by endocytosis and transport of CB1-gp into a compartment where the leupeptin-sensitive protease cleaves p57 to generate p42. This processing can also be inhibited by incubating cells at 12°C. Such low temperature chases could inhibit the action of the leupeptin-sensitive processing enzymes, transport to a compartment where processing occurs (M. J. Brickman, J. M. Cook and A. E. Balber, unpublished data), or both processes. The endocytic uptake and digestion of anti-VSG antibody from the surface of BF is strongly inhibited at 12°C (Russo et al., 1993).

The pulse-chase-biotinylation experiments show that more than 40% of all pulse labeled CB1-gp molecules appear on the surface before being exposed to the p57 cleaving enzyme. The biotinylated CB1-gp we recover from the surface after a 30 minute chase contains less than 10% p42; most of the core protein is unprocessed p57. The most straight-forward interpretation of this finding is that most of the newly synthesized CB1-gp on the surface after a 30 minute chase travels directly from the Golgi to the surface without passing through endosomes. Another interpretation is that some newly synthesized CB1-gp molecules pass through a recycling endosomal compartment between the Golgi and the surface. For this second explanation to be compatible with the observation that little p42 is present in the surface CB1-gp at 30 minutes of chase, one must postulate that newly synthesized CB1-gp molecules are not processed to yield p42 in the putative recycling endosome. This, in turn, implies either that the putative recycling endosome has different activities from the processing endosome that CB1-gp enters after leaving the surface, or that the rate of CB1-gp transport from the recycling endosome to the surface is faster than the rate of processing in this compartment. Our finding that p42 is present in the fraction of immunoprecipitated CB1-gp that did not bind to avidin beads is consistent with the idea that a direct Golgi to endosome pathway could operate along with the Golgi to surface route. However, this observation is difficult to interpret since CB1-gp molecules that appeared on the surface but were not biotinylated under the conditions we used, CB1-gp molecules that were in transit to or from the surface at the time of biotinylation, and CB1-gp molecules that were processed without reaching the surface, would all be present in this fraction. Thus, our experiments show that very few newly synthesized CB1-gp molecules reside in a processing endosome long enough for their p57 core protein to be converted to p42 before they reach the surface. We infer from this that the Golgi to surface to endosome route is a major CB1-gp trafficking pathway in BF.

Evidence that CB1-gp can recycle to the surface after being proteolytically processed was obtained when BF were surface biotinylated 60 minutes after being pulse labeled. A significant amount of p42 was detected in biotinylated surface CB1-gp under these conditions. If p57 is cleaved to p42 intracellularly, biotinylated CB1-gp containing p42 had to be exposed to the proteases inside the cell before arriving on the surface. In principle, this could occur in two different ways. First, molecules that reach the proteolytic compartment by the direct pathway could subsequently go to the surface; as discussed in the last paragraph, this is unlikely to be a quantitatively important pathway under the conditions we used for this experiment. Alternatively, CB1-gp molecules that travel from the surface to the proteolytic compartment could subsequently recycle to the surface. Other workers have suggested that VSG taken up from the FP in endocytic vesicles recycles from an intracellular compartment (Seyfang et al., 1990; see review by Duszenko and Seyfang, 1994), perhaps from collecting tubules (Webster and Grab, 1988). Coppens et al. (1993) found that chloroquine and monesin alter surface lipoprotein binding and interpreted their results to indicate that the lysoosmotrophic agents disrupt lipoprotein receptor recycling from acidic compartments. However, our data concerning CB1-gp is the only direct biochemical evidence that an invariant protein can travel from endosomes or lysosomes to the surface of trypanosomes.

Since CB1-gp can only be detected on the surface in the FP of BF (Brickman and Balber, 1993), and cell surface biotinylation occurs preferentially in the FP, CB1-gp probably reaches the surface in the FP. It may be that the Golgi to surface transport route for LGPs is particularly prominent in highly polarized cell types such as trypanosomes. As already noted, a very high proportion of AC17 molecules use this route in highly polarized MDCK cells; LGPs are targeted preferentially to the basolateral surface of these cells (Nabi et al., 1991).

Pathogenic trypanosomes diverged from other organisms very early in eucaryotic evolution (Sogin et al., 1989). Nevertheless, our results show that the Golgi to surface to lysosome transport route for distributing lysosomal membrane proteins exists in trypanosomes as well as in vertebrate cells. In addition to LGPs, mammalian acid phosphatase reaches lysosomes in endocytic vesicles derived from the cell surface and is subsequently processed to yield a soluble protein (Braun et al., 1989; Waheed et al., 1988). *Leishmania* also synthesize a membrane-bound acid phosphatase (Menz et al., 1991). Acid phosphatase is present in the FP as well as in endosomal and lysosomal compartments of trypanosomes (Schell et al., 1990; Langreth and Balber, 1975). Balber (1990) suggested that acid phosphatase and perhaps other trypanosome lysosomal hydrolases might pass through the FP on the way to endosomes and lysosomes. This evolutionary conservation of the cell surface to lysosome route of transport of lysosomal proteins suggests that this is a functionally important pathway in eucaryotes. It is interesting in this regard that pathways for targeting lysosomal hydrolases to lysosomes may be different in trypanosomatids and vertebrates, for *Trypanosoma cruzi* does not appear to use mammalian-type mannose-6-phosphate receptors for direct targeting of lysosomal enzymes from the *trans*-Golgi (Cazzulo et al., 1990).

The appearance of lysosomal membrane proteins and perhaps other proteins destined for intracellular compartments on the cell surface poses a potential threat to BF of pathogenic trypanosomes. These parasites depend on antigenic variation to survive in an immunocompetent host (Cross, 1990). For antigenic variation to be an effective immune evasion strategy, the VSG coat must shield invariant epitopes on functionally important surface proteins from contact with immune effector molecules such as antibodies. The surface coat does appear to be an effective barrier on much of the cell surface. However, invariant epitopes are exposed in the FP membrane (reviewed by Balber, 1990), and we show here that the FP is biotinylated more readily than the rest of the BF surface. The data presented in this paper shows that newly synthesized lysosomal membrane proteins are among the epitopes exposed in the FP.

Our studies also suggest that trypanosomes have evolved a way of dealing with this threat. CB1-gp only appears on the

surface in the FP membrane, and this membrane turns over rapidly through endocytosis. Even if antibodies to exposed epitopes enter the FP and combine with antigens in the FP membrane, the resulting immune complexes will be rapidly cleared from the surface into endosomes. Thus, the FP membrane is specialized to receive membrane proteins from the Golgi, to send some of these membrane proteins to lysosomes by endocytosis, and to clear the surface of immune complexes. It also follows that a mechanism exists in the FP to differentiate CB1-gp molecules that are included in endocytic vesicles from other proteins that are retained on the surface. Seen in this light, the highly specialized FP membrane plays a critical role in membrane traffic and in immune evasion by these parasites.

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